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13. ABSTRACT (Maximum 200 Words)

DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase involved in various DNA metabolism and damage signaling pathways. DNA damage activates DNA-PK, which in turn phosphorylates a number of key proteins involved in replication, repair, and transcription. Accordingly, DNA-PK has long been suspected as a factor involved in sensing and transmitting DNA damage signals to the downstream target, which eventually contributes to the genomic stability and prevention of cancer. The overall goal of this proposal is to explore the role of DNA-PK in the development and progression of breast cancer. Since DNA-PK is a DNA repair factor as well as involved in damage signaling pathway, levels of DNA-PK activity among breast cancer would contribute to their drug resistance and also provide the basis for selection of patients for treatment with chemotherapy drugs.

From the three-year study funded by DOD, we concluded that a peptide-based inhibitor preventing DNA-PKcs from forming a complex with Ku70/Ku80 significantly lowered DNA-PK activity. Furthermore, treatment of these breast cancer cells with target peptide significantly lowered the cell growth only in the presence of ionizing radiation, indicating that the peptide-based inhibitor exhibited a positive effect of on lowering drug resistance by specifically targeting DNA-PK *in vivo*. This result also validates a physiologic role for DNA-PK in chemotherapy drug resistance of breast cancers. Funding from the DOD breast cancer research also allowed us to study a potential role for DNA-PK in damage-induced cell cycle arrest, which was published recently.

14. SUBJECT TERMS
Damage-induced cell cycle checkpoint, DNA repair,DNA replication, S-phase arrest

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INTRODUCTION:

The overall goal of this proposal is to explore the role of DNA-dependent protein kinase (DNA-PK) in the development and progression of breast cancer. DNA-PK is a nuclear serine/threonine protein kinase involved in various DNA metabolism and damage signaling pathways. DNA damage activates DNA-PK, which in turn phosphorylates a number of key proteins involved in replication, repair, and transcription. Accordingly, DNA-PK has long been suspected as a factor involved in sensing and transmitting DNA damage signals to the downstream target, which eventually contributes to the genomic stability and prevention of cancer. To see a correlation between DNA-PK activity and different stages of cancer, DNA-PK activity levels of various stages of breast cancer will be analyzed. Outcome of this study will provide us the information as to whether DNA-PK can be used as a prognostic measure of breast cancer progression. We will also analyze breast cancer cells grown in culture and grown as tumor grafts in mice to see whether DNA-PK plays a role in chemotherapy resistance. A strong correlation between DNA-PK activity and drug resistance of breast cancers would provide the basis for selection of patients for treatment with chemotherapy drugs. Furthermore, the information regarding the role of DNA-PK in drug resistance would be very useful for drug discovery aimed at increasing the sensitivity of tumors to chemotherapy.

BODY:

Task 1. To characterize the relationship between DNA-PK and breast cancer development/ progression (months 1-36)

In order to assess DNA-PK activity and its expression in various stages of breast cancer cells, we obtained several tissue samples from the Indiana University Cancer Center (IUCC) Tumor Bank and carried out a preliminary study. The number of breast cancer tissues we tested, however, were only 5 samples that were not enough to make any conclusion on the relationship between DNA-PK and breast cancer development/progression. We had some difficulty in obtaining enough number of specimens for different stages of breast cancer by the FIGO (Federation of International Gynecologic Organization) guideline, however the IU Cancer Center strongly supports our study and provided enough number of specimens to carry out our proposed study. This study is currently in progress. Once obtained, tissue samples will be grown in tissue culture dish (25 x 150 mm) and cell extracts will be prepared for measurement of DNA-PK activity. Also, molecular analysis of the alteration of DNA-PK activity in breast cancers will follow to evaluate the relationship between DNA-PK activity and the degree status of breast cancer.

Task 2. To determine whether DNA-PK plays a role in DNA repair and/or the chemotherapy drug resistance among breast cancers (months 1-36)

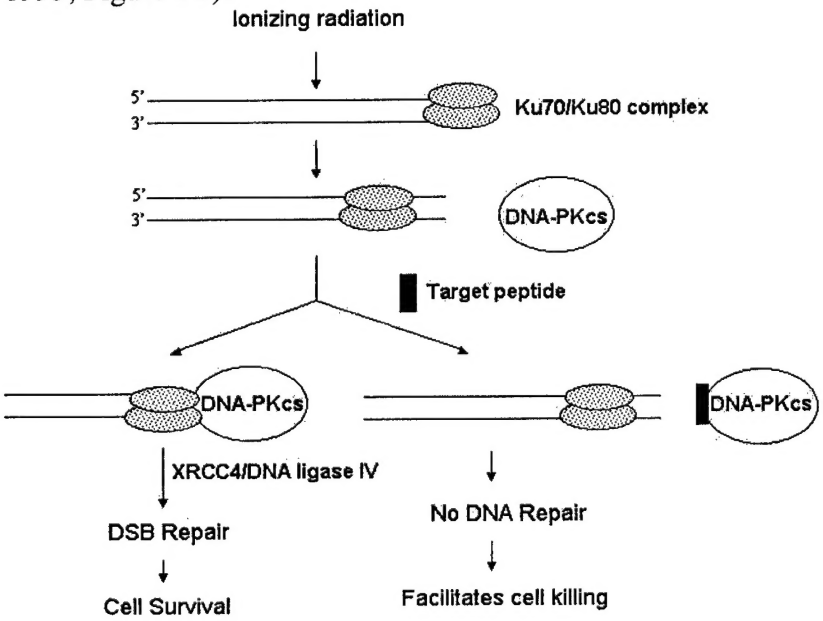
DNA-PK activity and chemotherapy drug resistance of breast cancer cells (months 6-24):

We initially proposed to determine whether altered DNA-PK activity in breast cancers is associated with chemotherapy drug. To see a relationship between DNA-PK and drug resistance of breast cancer cells, we prepared a peptide-based inhibitor that binds to the C-terminus of Ku80 (Gell & Jackson, 1999) and competitively inhibits DNA-PKcs from binding to Ku70/Ku80. We tested these peptides for their effects on DNA-PK kinase activity *in vitro* using a specific substrate peptide (EPPLSQEAFADLWKK) (Lees-Miller *et al*, 1990). The target peptide (HNI-38) effectively inhibited DNA-PK activity under the conditions where the control peptide (HN-26) had no effect, indicating that HNI-38 prevented DNA-PKcs from forming a complex with Ku70/Ku80 (Figure 2).

A previous study indicates that DNA-PK mutant cells exhibit sensitivity to irradiation and cisplatin treatment (Britten *et al*, 1999; Frit *et al*, 1999). Also, studies with drug-resistant and sensitive cells indicate that higher levels of DNA-PK expression lead to drug-resistant cells, whereas the low DNA-PK activity was associated with cells with drug-sensitive phenotype (Shen *et al*, 1997). Since the target peptide effectively interferes with DNA-PK activity, we attempted to analyze whether alteration of DNA-PK activity induced by treatment with peptide-based inhibitor affected drug resistance of breast cancer cells (Figures 3-5).

Targeted Inhibition of DNA-PK

Ku70 and Ku80 form a heterodimeric complex that is important for DNA-termini binding; neither Ku70 nor Ku80 alone is active in DNA binding activity (Wu and Lieber, 1996; Gell and Jackson, 1999). The C-terminus of both Ku70 and Ku80 are necessary for heterodimer assembly as well as for DNA-termini binding (Wu and Lieber, 1996; Gell and Jackson, 1999). A recent protein interaction study indicated that DNA-PKcs interacting domain is localized at the extreme C-terminus of Ku80 (amino acids 720-732) (Gell and Jackson, 1999). Since the C-terminus of Ku80 is also likely involved in heterodimer assembly and DNA termini binding, this region (amino acids 720-732 of Ku80) was selected to synthesize a target peptide that would prevent DNA-PKcs from binding to Ku70/Ku80 regulatory subunits (see Figure 1A). To deliver a peptide to the cancer cells, a cell-permeable peptide import domain and the nuclear localization domain were added to the target peptide to obviate the need for permeabilization or microinjection of individual cells (Lin et al., 1995; Figure 1B).



HNI-38: AAVALLPAVLLALLAP**VQRKRQKLM***Y*

HN-26: AAVALLPAVLLALLAP**VQRKRQKLM***Y*

HI-29: AAVALLPAVLLALLAP*Y*

NI-22: **VQRKRQKLM***Y*

Figure 1A (upper). A peptide co-therapy strategy for targeted inhibition of DNA-PK in cancer cell co-therapy. Treatment of cells with ionizing radiation (or chemotherapy drug) induces strand-break DNA

damage. To repair DNA damage, DNA-PK heterotrimeric complex (Ku70, Ku80, and DNA-PKcs) needs to be assembled at the ends of DNA. Target peptide representing amino acids 720-732 of Ku80 not only interacts with DNA-PKcs, but may also be involved in Ku heterodimer assembly and DNA termini binding. As a result, cells treated with target peptide will exhibit poor or no DNA repair and become highly sensitive to ionizing radiation or chemotherapy drug. **1B (lower).** The Sequences of synthetic peptide-based inhibitor and control peptides (single-letter amino acid code). Membrane-translocating hydrophobic signal sequence is underlined and the nuclear localization sequence is shown in **bold face**. Twelve residue of peptide inhibitor region is indicated as bar ( ) at the C-terminus. The tyrosine residue (*Y*) is included for ^{125}I-labeling to determine the import efficiency of the peptide into the cells. Peptide-based inhibitor contains the hydrophobic region localization sequence, so-called membrane-translocating carrier, which not only facilitates secretion of proteins, but also is important for importing synthetic peptides into the cell (Lin YZ *et al*, 1995). This localization peptide is capable of carrying a functional domain such as nuclear localization signal (NLS) (Boulikas T, 1994). We therefore synthesized a 38-residue peptide (HNI-38) comprising the signal peptide sequence (AAVALLPAVLLALLAP) and NLS (VQRKRQKLM) followed by a tyrosine (Y) residue and 12-residue of peptide inhibitor sequence (EGGDVDDLLDMI) representing the C-terminus of Ku80 (amino acids #721-732). The tyrosine residue was used for ^{125}I-labeling to determine the import efficiency of synthetic peptides into the cells (and nuclei) (Lin YZ *et al*, 1995).

A target peptide interrupts the interaction between DNA-PKcs and Ku70/Ku80 as well as the binding of Ku complex to DNA. DNA-PKcs and Ku70/Ku80 are abundant proteins approximately 5 x 10^5 molecules per human cells (Lee and Kim, 2002) and most of Ku70/Ku80 heterodimer exists in cell extracts without forming a complex with DNA-PKcs in the absence of DNA (Hammarsten and Chu, 1998). Therefore, target peptide (HNI-38) was analyzed for its effect on interaction between DNA-PKcs and Ku70/Ku80 in the presence of dsDNA. Varying concentrations of either control (HN-26) or target peptide (HNI-38) was incubated with cell extracts containing DNA-PKcs and Ku complex in the presence of dsDNA cellulose, and examined for its effect on binding of Ku complex and DNA-PKcs to DNA following the dsDNA cellulose pulldown assay (Figure 2A). Although it was marginal, the addition of increasing amount of target peptide (HNI-38) not control peptide (HN-26) led to a decrease in DNA-PKcs associated with dsDNA, suggesting that target peptide binds to DNA-PKcs and inhibits its binding to Ku70/Ku80. It is also noted that the addition of target peptide affected the binding of Ku70/Ku80 to the dsDNA cellulose (Figure 2A).

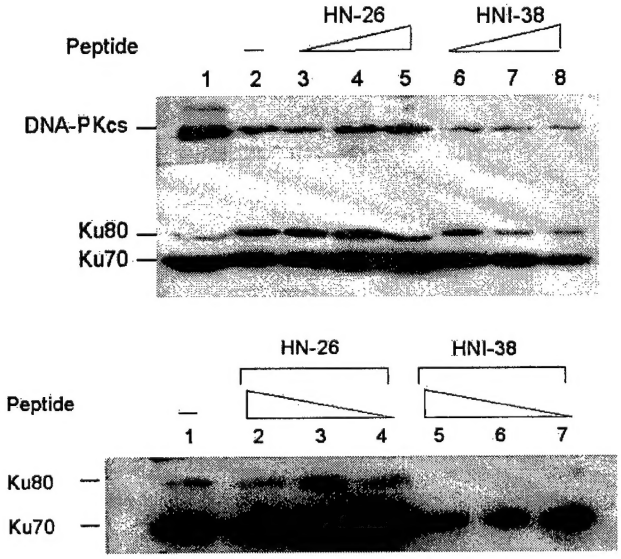


Figure 2. Effect of the target peptide on interaction of Ku70/Ku80 with DNA-PK or with dsDNA. **Panel A (upper).** The target peptide (HNI-38) interferes with association of DNA-PKcs with dsDNA. Partially purified DNA-PK fraction (100 ng) was incubated with 0 nM (lane 2), 10 nM (lanes 3 & 6), 50 nM (lanes 4 & 7), and 100 nM (lanes 5 & 8) of either control peptide or target peptide prior to dsDNA cellulose pull-down assay. Lane 1 contained partially purified DNA-PK without dsDNA pulldown assay. The protein-dsDNA cellulose complex was analyzed by the procedure described in Methods section. **Panel B (lower).** Effect of HNI-38 on DNA binding activity of Ku70/Ku80 complex. Purified Ku70/Ku80 complex (100 ng) was incubated with 10 nM (lanes 4 & 7), 50 nM (lanes 3 & 6), and 100 nM (lanes 2 & 5) of either control peptide or target peptide prior to dsDNA cellulose pull-down assay. Following the dsDNA cellulose pull-down, Ku70 and Ku80 were analyzed by 10% SDS-PAGE and Western blot.

To further examine the effect of HNI-38 on Ku's DNA binding activity, target peptide was incubated with purified Ku70/Ku80 complex in the presence of dsDNA cellulose, and the reaction mixtures were analyzed for the presence of Ku70 and Ku80 following the dsDNA pulldown assay (Figure 2B). In keeping with Figure 2A, target peptide (HNI-38) significantly interfered with binding of Ku complex to dsDNA under the conditions where control peptide (HN-26) showed virtually no effect (Figure 2B). This result suggests that target peptide not only affects the interaction between DNA-PKcs and Ku, but also interferes with Ku's DNA binding activity.

Effect of target peptide (HNI-38) on DNA-PK kinase activity

Interaction of DNA-PKcs with Ku complex are necessary for activation of its kinase activity (Gottlieb and Jackson, 1993; Hartley et al., 1995), therefore, the efficacy of target peptide was analyzed by measuring DNA-PK kinase activity *in vitro* in the presence of either HI-26 or HNI-38. DNA-PK kinase activity was inhibited up to 50% in the presence of HNI-38 under the conditions where a control peptide (HI-26) showed minimal effect (Figure 3), strongly supporting a notion that target peptide specifically binds to DNA-PKcs and interferes with interaction between DNA-PKcs and Ku complex. Inhibitory effect of target peptide on DNA-PK occurred at low peptide concentration (>20 μM) and, in the presence of 20μM or higher, both target and control peptides inhibited DNA-PK activity (data not shown).

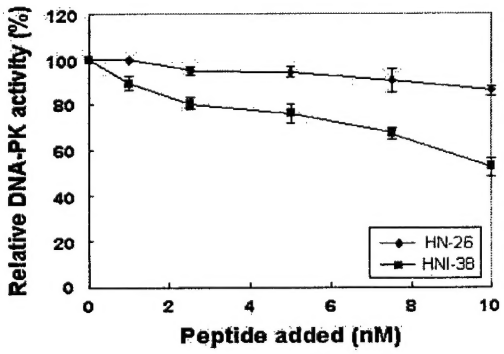


Figure 3. Effect of target peptide on DNA-PK kinase activity *in vitro*. Partially purified DNA-PK fraction was incubated with various concentrations of either control peptide or target peptide prior to the addition of substrate peptide and other components for DNA-PK kinase assay. DNA-PK activity was measured as the relative amounts of ^{32}P transferred to the substrate peptide.

Target peptide interferes with repair of double-stranded DNA breaks induced by IR

IR-induced double-stranded DNA breaks are efficiently repaired by non-homologous end-joining (NHEJ) process. Genetic and biochemical studies strongly indicated that DNA-PK plays an

essential role in NHEJ (Jeggo, 1998; Jin et al., 1997; Blunt et al., 1995). Hence, an alternative way to determine the efficacy of peptide inhibitor is to measure the repair of double-stranded DNA breaks following IR. Breast cancer cells (NCI) grown in the presence of [^{14}C]-thymidine (DiBiase et al, 2000) were treated with either a control or target peptide for 24 hrs. Following irradiation (40 Gy), cells were harvested at various time points and intact chromosomal DNA and DSBs were separated by pulsed field gel electrophoresis (0.5% agarose). Treatment of NCI cells with IR (40 Gy) induced substantial amounts of dsDNA breaks, most of which were repaired within 4 hrs. Cells treated with target or control peptide did not show any difference in generating DSBs following IR (Figure 4A; lane 2 vs. lanes 8 & 14). On the other hand, cells treated with target peptide (Figure 4A; lanes14-18) compared with those treated with control peptide (Figure 4A; lanes 8-12) showed a noticeable decrease in DSB repair activity. This result suggests that target peptide interfered with dsb repair *in vivo* through the targeted inhibition of DNA-PK activity.

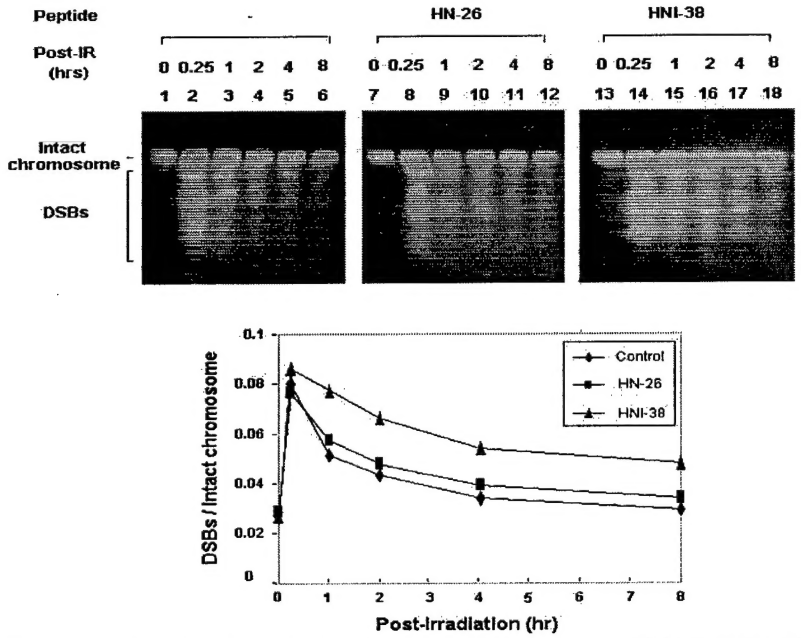


Figure 4. Effect of target peptide on double-stranded DNA breaks (dsb) repair. Breast cancer cells (NCI) grown in ^{14}C-containing media were treated with ionizing radiation (40 Gy) in the presence of no peptide (lanes 1-6), 50 nM of control peptide (lanes 7-12), or 50 nM of target peptide (lanes 13-18). After harvesting the cells at various time points, intact chromosomes and double-stranded DNA breaks (DSBs) were separated by gel electrophoresis (**upper panel**: fluorography) and were quantified by liquid scintillation counter (**lower panel**).

Target peptide inhibits breast cancer cell growth only in the presence of DNA damage

Cells lacking DNA-PK catalytic subunit showed increased sensitivity to DNA damaging drugs or ionizing radiation (IR) (Lees-Miller et al., 1995; Kirchgessner et al., 1995), suggesting that DNA-PK activity is essential for DNA repair and cell survival upon DNA damage. We therefore tested whether a targeted inhibition of DNA-PK by a peptide HNI-38 would sensitize breast cancer cells upon treatment of ionizing radiation or chemotherapeutic drug (cisplatin). Two breast cancer cells (NCI and MDA231) were treated with either control (HI-26) or target peptide (HNI-38) and tested for the efficacy of DNA-PK inhibitory peptide on lowering resistance of cells in response to ionizing radiation using standard colony count cell survival assay. Both control and target peptides did not

show any effect on cell growth in the absence of ionizing radiation. However, cells treated with IR showed significant cell growth inhibition in the presence of target peptide but not with control peptide (Figure 5A), suggesting that cell growth inhibition by target peptide occurs through targeting DNA-PK activity. Cells treated with cisplatin, although not as effective as those treated with IR, also showed inhibitory effect on cell growth in the presence of HNI-38 (Fig 5B).

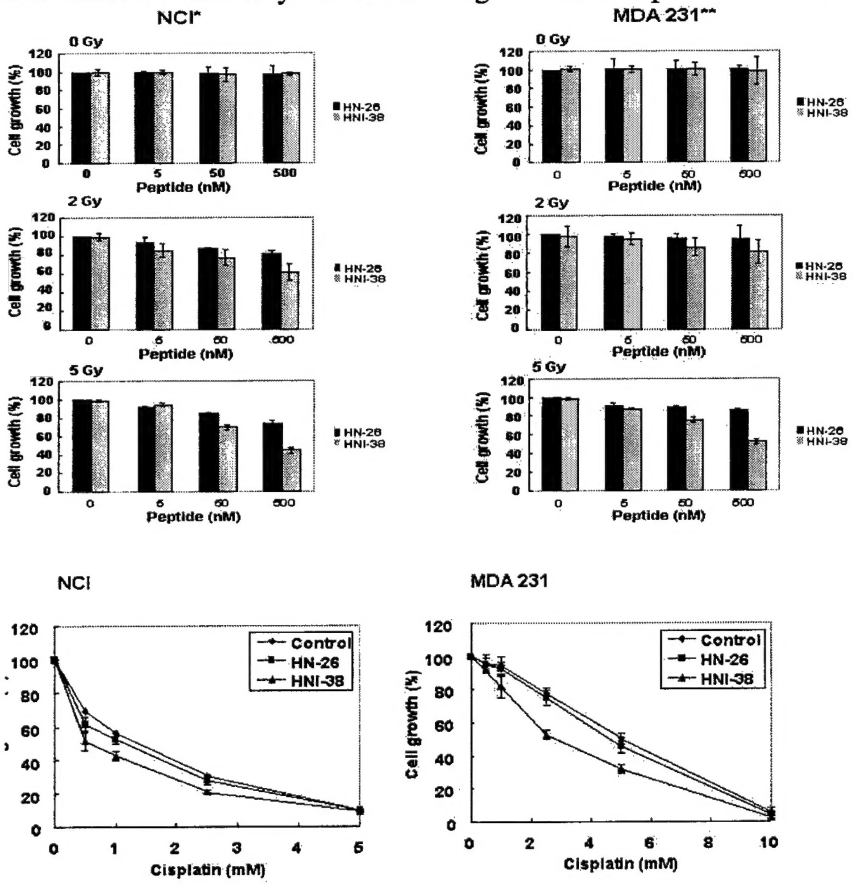


Figure 5. Effect of control (HN-26) and target (HNI-38) peptides on the growth of breast cancer cells treated with ionizing radiation (**upper panel**) or cisplatin (**lower panel**). Values expressed are means (+ S.E.) of the three replications (*, $p < 0.01$; ** $p < 0.01$). The clonogenic assay was used for the cells treated with ionizing radiation and the cell survival assay (MTT) was employed for those treated with cisplatin (see the Methods section for the detailed procedure).

In summation, DNA-PK activity is essential for DNA repair as well as cell cycle arrest in response to DNA damage, which contributes to cell survival by protecting cells from apoptosis. Cells treated with target peptide not control peptide showed a noticeable decrease in DSB repair following high dose (40 Gy) of IR, suggesting that HNI-38 specifically targets DNA-PK *in vivo* and interferes with dsb repair activity through inhibition of DNA-PK activity. Targeted inhibition of DNA-PK by HNI-38 also caused cell growth inhibition only when cells were treated with IR, suggesting that HNI-38 targeted DNA-PK and lowered resistance of cells in response to ionizing radiation, which eventually causes growth inhibition of both NCI and MDA231. Treatment of cells with HNI-38 also showed additive effect on cell growth inhibition in response to cisplatin treatment. This observation is in keeping with previous findings that DNA-PK is directly involved in NER action in mammals (Muller et al., 1998). It also supports a notion that a targeted inhibition of DNA-PK would sensitize

cancer cells upon treatment of chemotherapeutic drugs such as cisplatin. Together, our study described here not only validates DNA-PK as a useful molecular target for the treatment of drug-resistant cancer cells, but also supports physiologic role for DNA-PK in IR or chemotherapy drug resistance of cancer cells.

KEY RESEARCH ACCOMPLISHMENTS:

1. Demonstration of the relationship between DNA-PK activity and drug resistance of breast cancer cells

2. Validation of a peptide-based inhibitor of DNA-PK on lowering the growth of breast cancer cells following radiation treatment

3. Basic research on the role for DNA-PK in DNA repair and damage-induced cell cycle arrest

REPORTABLE OUTCOMES: (works supported by DAMD 17-00-1-0295)

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CONCLUSIONS:

The overall goal of this proposal is to explore the role for DNA-PK in the development and progression of breast cancer. Since DNA-PK is a key DNA repair factor as well as involved in damage signaling pathway, levels of DNA-PK activity among breast cancer would contribute to their drug resistance and also provide the basis for selection of patients for treatment with chemotherapy drugs.

From the three-year of study, we concluded that a peptide-based inhibitor preventing DNA-PKcs from forming a complex with Ku70/Ku80 significantly lowered DNA-PK activity. Furthermore, treatment of these breast cancer cells with target peptide significantly lowered the cell growth only in the presence of ionizing radiation, indicating that the peptide-based inhibitor exhibited a positive effect on lowering drug resistance by specifically targeting DNA-PK *in vivo*. This result also validates a physiologic role for DNA-PK in chemotherapy drug resistance of breast cancers.

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APPENDICES:

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A Targeted Inhibition of DNA-Dependent Protein Kinase Sensitizes Breast Cancer Cells Following Ionizing Radiation

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ABSTRACT

A major mechanism by which cancer cells become resistant to ionizing radiation (IR) and chemotherapy drugs is by enhanced DNA repair of the lesions; therefore, through inhibition of DNA repair pathways that tumor cells rely on to escape chemotherapy, we expect to increase the killing of cancer cells and reduce drug resistance. DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase essential for DNA repair as well as sensing and transmitting a damage signal to downstream targets leading to cell cycle arrest. We used a peptide cotherapy strategy to see whether a targeted inhibition of DNA-PK activity sensitizes breast cancer cells in response to IR or chemotherapy drug. A synthesized peptide representing the C terminus of Ku80 (HNI-38) selectively targeted and disrupted interaction between Ku complex and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) as well as the DNA binding activity of Ku that led to the inhibition of DNA-PK activity and reduction in double-stranded DNA break (dsb) repair activity. Furthermore, a peptide-based inhibitor with target sequence effectively inhibited the growth of breast cancer cells only in the presence of DNA damage, suggesting that the target peptide sensitizes cancer cells through blocking dsb DNA repair activity. Together, this study not only validates the involvement of the C terminus of Ku80 in Ku's DNA termini binding and interaction with DNA-PKcs, but also a supports physiological role for DNA-PK in IR or chemotherapy drug resistance of cancer cells.

DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase composed of 460-kDa catalytic subunit (DNA-PKcs) and a heterodimer of Ku70 and Ku80, which act as a DNA binding and regulatory component for the complex (Gottlieb and Jackson, 1993; Jin et al., 1997; Lieber et al., 1997). DNA-PK is a key component of the nonhomologous end joining (NHEJ) pathway and V(D)J recombination (Blunt et al., 1995), with the unique property of being activated by DNA ends (Critchlow and Jackson, 1998; Jeggo, 1998; Featherstone and Jackson, 1999). It has long been suspected as a factor involved in sensing and transmitting DNA damage signals to the downstream targets (Jackson, 1997; Weaver and Alt, 1997; Lee and Kim, 2002). Previous studies demonstrated that DNA-PK is necessary for activation of p53 (Woo et al., 1998), nucleotide excision repair (Muller et al., 1998), and damage-induced S-phase arrest (Park et al., 1999) in response to DNA damage, all of which contribute to cell protection from genetic alterations as well as chemotherapy drug resistance. In vivo observations indicated that DNA-PK mutant cells exhibited sensitivity to ionizing irradiation and chemotherapy drugs and were associated with lower DNA repair activity following DNA damage, suggesting a positive role for DNA-PKcs in DNA repair (Britten et al., 1999; Frit et al., 1999). Also, studies with drug-resistant or drug-sensitive cancer cells suggested that higher levels of DNA-PK expression lead to drug-resistant cells, whereas low DNA-PK activity was associated with cells with drug-sensitive phenotype (Shen et al., 1997) and was linked to cell death via the accumulation of damaged DNA.

The current model of DNA-PK complex activation by DNA is based on the tenet that without DNA, DNA-PKcs is inactive and incapable of binding Ku (Hanawalt, 1994; Suwa et al., 1994; Hartley et al., 1995). When a double-strand break is introduced, Ku complex binds to the DNA because of its high affinity for DNA ends. The binding of Ku induces conformational change that allows it to interact with DNA-PKcs. It is unclear how the Ku/DNA complex activates the kinase activity of DNA-PKcs. One hypothesis is that DNA-PKcs undergoes a conformational change upon association with the Ku/DNA complex, and this conformational change accounts for the activation of kinase activity. The kinase activity associ-

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ABBREVIATIONS: DNA-PK, DNA-dependent protein kinase; DNA-PKcs, catalytic subunit of DNA-dependent protein kinase; NHEJ, nonhomologous end-joining; IR, ionizing radiation; dsb, double-stranded DNA break; dsDNA, double-stranded DNA; MTT, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide; PAGE, polyacrylamide gel electrophoresis.

ated with DNA-PK is needed for DNA repair in vivo, since expression of a kinase-inactive form of DNA-PKcs failed to complement the radiosensitive phenotype of a mammalian cell line lacking the DNA-PKcs protein (Kurimasa et al., 1999). However, the physiological targets of DNA-PK in vivo are still not clear. The DNA-PK complex can physically tether two ends of a dsb in close proximity in vitro, suggesting the hypothesis that the DNA-PK complex acts as a scaffold to assemble the NHEJ pathway proteins at a DSB (Cary et al., 1997).

We hypothesize that 1) DNA-PK plays an important role in conferring cells becoming resistant to ionizing radiation or anticancer DNA-damaging drugs, and 2) targeted inhibition of DNA-PK sensitizes drug resistance of cancer cells and facilitates cell killing. By developing peptides that can directly interfere with DNA-PK activity, one can develop a novel cotherapy that can selectively target and disrupt the IR-induced dsb repair pathway, which will enhance the efficacy of currently available treatments and also broaden the usefulness of chemotherapeutic agents in cancer treatment. We have therefore synthesized a peptide (HNI-38) mimicking the domain of Ku80 essential for interaction with its catalytic subunit (DNA-PKcs) and tested whether it can selectively target and disrupt DNA-PK activity required for dsb repair, which potentiates the effect of chemotherapy drug in cancer treatment. This strategy can be applied to cancer cotherapy, which will broaden the usefulness of chemotherapeutic agents in cancer treatment.

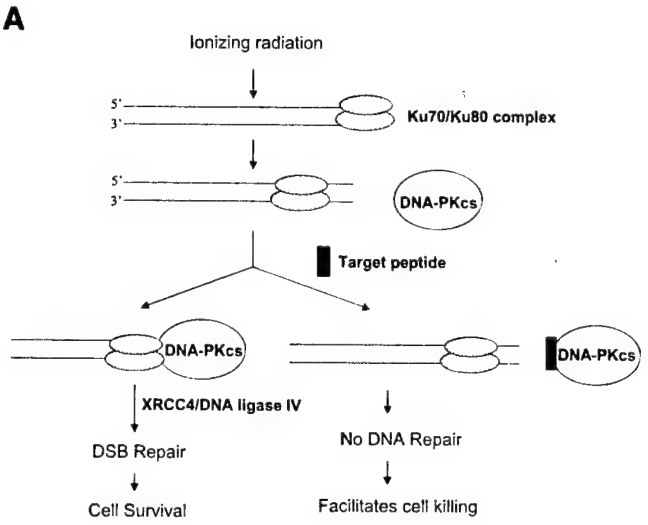
Materials and Methods

Cell Lines, Antibodies, and Chemicals. Two human breast cancer cells, MDA231 and NCI, were obtained from Dr. George Sledge (Indiana University Cancer Center, Indianapolis, IN) and maintained in minimal essential medium supplemented with 10% fetal bovine serum at 37°C in a CO_2 incubator. Antibodies to Ku70/80 and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) were obtained from either Upstate Biotechnology (Lake Placid, NY) or BD PharMingen (San Diego, CA). [γ-^{32}P]ATP (4500 Ci/mmol) was from ICN Pharmaceuticals (Costa Mesa, CA), and dsDNA cellulose and cisplatin were obtained from Sigma-Aldrich (St. Louis, MO).

dsDNA Cellulose Pull-Down Assay. The dsDNA cellulose fraction (100 μg) containing DNA-PKcs and Ku70/Ku80 heterodimer was prepared from HeLa cells (Lees-Miller et al., 1990) and incubated with the indicated amount of either control (HN-26) or a target (HNI-38) peptide in the presence of 4 mM ATP and 50 μl of dsDNA cellulose (3 mg of dsDNA/mg of cellulose; Sigma-Aldrich) for 3 h at 4°C with rocking for the interaction of DNA-PKcs and Ku70/Ku80 heterodimer. Where indicated, purified Ku70/Ku80 complex (100 ng) was used instead of the dsDNA cellulose fraction. After centrifugation at 4000 rpm, the precipitates were collected and washed three times with a buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) for protein analysis. For Western blot, the precipitates were separated by 8 or 10% SDS-PAGE, transferred to nitrocellulose (Millipore Corp., Bedford, MA), and blotted with primary antibody to Ku70/80 and/or DNA-PKcs followed by a peroxidase-coupled secondary antibody (Amersham Biosciences Inc.,, Piscataway, NJ) and an enhanced chemiluminescence (ECL kit, Amersham) reaction prior to visualization on Kodak-O-mat film.

DNA-PK Kinase Assay. Reaction mixtures (20 μl) contained 20 mM HEPES-KOH (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 7 mM $MnCl_2$, 5 mM NaF, 1 mM Na_3VO_4, 50 μl of [^{32}P]ATP, 150 μl of substrate peptide, 0.4 μg of DNase I-activated calf-thymus DNA (Sigma-Aldrich), and 100 ng of partially purified DNA-PK complex. DNA-PK complex was partially purified from HeLa cells according to the procedure described previously (Lees-Miller et al., 1990). Substrate peptide (EPPLSQEAFADLWKK) representing amino acids 11–24 of p53 was used as a substrate for DNA-PK assay (Lees-Miller et al., 1990). To find out whether the peptide interferes with DNA-PK kinase activity, various amounts of peptide inhibitor were added to the reaction. After incubation at 30°C for 30 min, the reaction mixtures were stopped with 30% acetic acid and a portion of the reaction mixtures (5 μl) was spotted onto a P81 strip, and after extensive washing, radioactivity was measured. DNA-PK activity was measured as picomoles of ^{32}P transferred to the substrate peptide.

Cell Survival Assay. Cells (1.0×10^4 cells/well) were seeded in a 96-well plate in the presence of control or target peptide and incubated for 24 h before the treatment of cells with either ionizing radiation or cisplatin. After further incubation at 37°C, 5% CO_2 for 72 h, cell survival was measured using a colorimetric cell survival assay from Roche Diagnostics (Indianapolis, IN; MTT Cell Proliferation Kit). Alternatively, clonogenic assay was used to measure the ability of cells to form colonies on 100-mm^2 tissue culture dishes after treatment with ionizing radiation or cisplatin. Controls consisted of cells untreated with peptides, cells untreated with DNA-damaging agent, or cells without either treatment. Cells were continuously exposed for 5 days to the indicated concentrations of the peptide and colonies were stained with crystal violet; then, colonies greater than 50 cells were counted. Each point represents mean values ± S.E.,



B

HN-26 *AAVALLPAVLLALLAP*VQRKRQKLMY

HNI-38 *AAVALLPAVLLALLAP*VQRKRQKLMYEGGDVDDLLDMI

Fig. 1. Panel A, a peptide cotherapy strategy for targeted inhibition of DNA-PK in cancer cell cotherapy. Treatment of cells with ionizing radiation (or chemotherapy drug) induces strand-break DNA damage. To repair DNA damage, DNA-PK heterotrimeric complex (Ku70, Ku80, and DNA-PKcs) needs to be assembled at the ends of DNA. Target peptide representing amino acids 720–732 of Ku80 not only interacts with DNA-PKcs, but may also be involved in Ku heterodimer assembly and DNA-termini binding. As a result, cells treated with target peptide will exhibit poor or no DNA repair and become highly sensitive to ionizing radiation or chemotherapy drug. Panel B, synthetic peptide used for cotherapy study. Sequences of synthetic peptide-based inhibitor and control peptides (single-letter amino acid code). The membrane-translocating hydrophobic signal sequence is indicated in italic letters and the nuclear localization sequence is shown in boldface. Twelve residues of the peptide inhibitor region are indicated at the C terminus of HNI-38.

each conducted with triplicate plates. The p values in Fig. 5A (see under *Results*) were obtained from two separate experiments using a one-way analysis of variance method (SigmaStat for Windows, version 2.03; SPSS Science, Chicago, IL).

Double-Stranded DNA Break (dsb) Repair Assay. Kinetics of rejoining of radiation-induced damaged DNA in breast cancer cells following exposure of cells to 40 Gy gamma irradiation (^{137}Cs) were measured by pulsed field gel electrophoresis. Breast cancer cells (NCI) were grown in the presence of 2.5 μM [^{14}C]thymidine (0.1 μCi/ml) (DiBiase et al., 2000) and treated with either a control or target peptide. After irradiation (40 Gy), cells were further incubated at 37°C with prewarmed (42°C) fresh medium to allow DSB repair, and then harvested at various times and resuspended in serum-free medium at a concentration of 2 to 5 $\times$ 10^6 cells/ml. Cells were mixed with an equal volume of 1% agarose, and the solidified cell-agarose suspension was lysed with buffer containing 10 mM Tris (pH 8.0), 50 mM NaCl, 0.5 M EDTA, 2% *N*-lauryl sarcosyl, and proteinase E and O (0.1 mg/ml) for 16 to 18 h at 50°C (DiBiase et al., 2000). DNA double-strand breaks were analyzed by asymmetric field inversion gel electrophoresis using 0.5% agarose gel in 0.5× Tris borate-EDTA at 10°C for 40 h. After electrophoresis, gels were analyzed by fluorography. For quantification of damaged DNA repair, intact chromosome and damaged DNA were separately removed from the gel and measured for ^{14}C labeling using a liquid scintillation counter.

Results

Targeted Inhibition of DNA-PK. Ku70 and Ku80 form a heterodimeric complex that is important for DNA-termini binding; neither Ku70 nor Ku80 alone is active in DNA binding activity (Wu and Lieber, 1996; Gell and Jackson, 1999). The C termini of both Ku70 and Ku80 are necessary for heterodimer assembly as well as for DNA-termini binding (Wu and Lieber, 1996; Gell and Jackson, 1999). A recent protein interaction study indicated that the DNA-PKcs interacting domain is localized at the extreme C terminus of Ku80 (amino acids 720–732) (Gell and Jackson, 1999). Since the C terminus of Ku80 is also likely involved in heterodimer assembly and DNA-termini binding, this region (amino acids 720–732 of Ku80) was selected to synthesize a target peptide that would prevent DNA-PKcs from binding to Ku70/Ku80 regulatory subunits (see Fig. 1A). To deliver a peptide to the cancer cells, a cell-permeable peptide import domain and the nuclear localization domain were added to the target peptide to obviate the need for permeabilization or microinjection of individual cells (Lin et al., 1995; Fig. 1B).

A Target Peptide Interrupts the Interaction between DNA-PKcs and Ku70/Ku80 as well as the Binding of Ku Complex to DNA. DNA-PKcs and Ku70/Ku80 are abundant proteins, approximately 5 $\times$ 10^5 molecules per human cell (Lee and Kim, 2002, and references therein), and most of the Ku70/Ku80 heterodimer exists in cell extracts without forming a complex with DNA-PKcs in the absence of DNA (Hammarsten and Chu, 1998; Fig. 2A). Therefore, target peptide (HNI-38) was analyzed for its effect on interaction between DNA-PKcs and Ku70/Ku80 in the presence of dsDNA. Varying concentrations of either control (HN-26) or target peptide

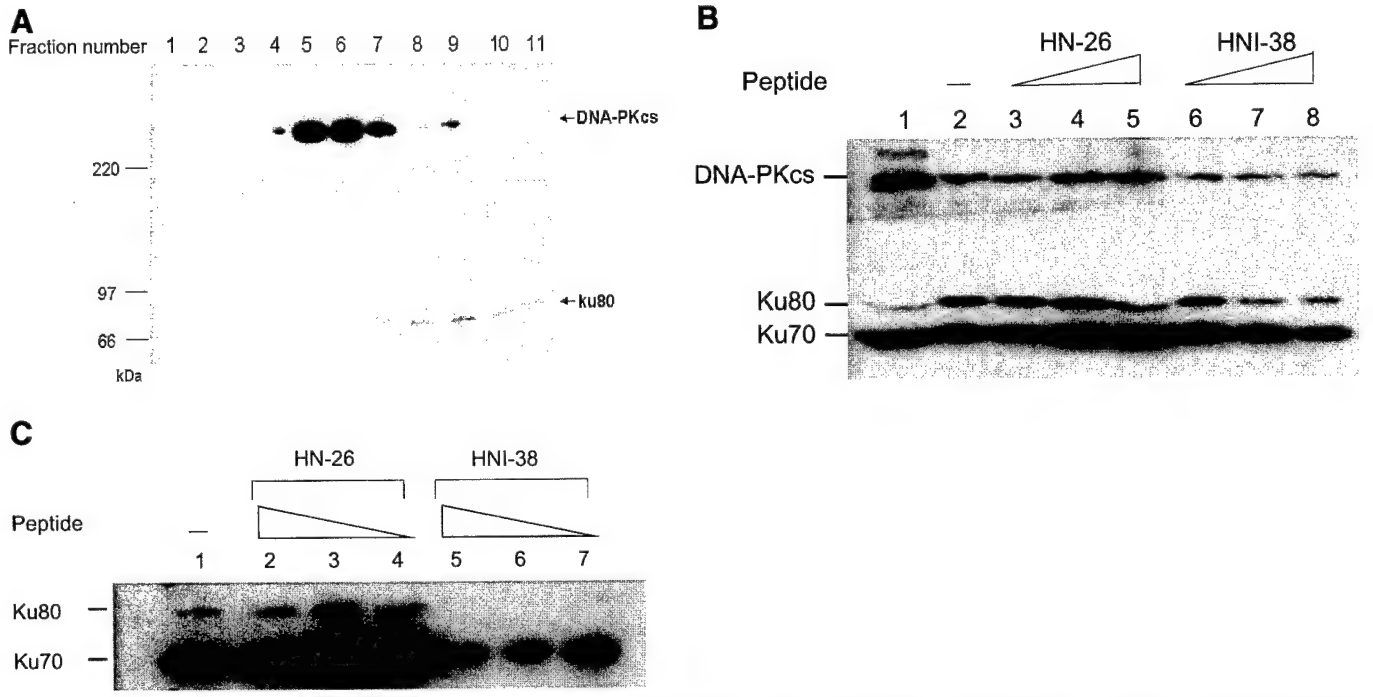


Fig. 2. Effect of the target peptide on interaction of Ku70/Ku80 with DNA-PK or with dsDNA. Panel A, Ku70/Ku80 complex is not in complex with DNA-PKcs. Chromatographic separation of DNA-PKcs from Ku70/Ku80 heterodimer. Partially purified DNA-PK fractions (dsDNA cellulose fraction; see Lees-Miller et al., 1990 for details) were subjected to heparin-Sepharose column chromatography and eluted with 100 to 500 mM NaCl gradient. Fractions were analyzed by 6% SDS-PAGE for DNA-PKcs and Ku80 followed by immunoblot using anti-DNA-PKcs and anti-Ku80 antibodies. Panel B, the target peptide (HNI-38) interferes with association of DNA-PKcs with dsDNA. Partially purified DNA-PK fraction (100 ng) was incubated with 0 nM (lane 2), 10 nM (lanes 3 and 6), 50 nM (lanes 4 and 7), and 100 nM (lanes 5 and 8) concentrations of either control peptide or target peptide prior to dsDNA cellulose pull-down assay. Lane 1 contained partially purified DNA-PK without dsDNA pull-down assay. The protein-dsDNA cellulose complex was analyzed by the procedure described under *Materials and Methods*. Panel C, effect of HNI-38 on DNA binding activity of Ku70/Ku80 complex. Purified Ku70/Ku80 complex (100 ng) was incubated with 10 nM (lanes 4 and 7), 50 nM (lanes 3 and 6), and 100 nM (lanes 2 and 5) concentrations of either control peptide or target peptide prior to dsDNA cellulose pull-down assay. After the dsDNA cellulose pull-down, Ku70 and Ku80 were analyzed by 10% SDS-PAGE and Western blot.

(HNI-38) were incubated with cell extracts containing DNA-PKcs and Ku complex in the presence of dsDNA cellulose, and examined for its effect on binding of Ku complex and DNA-PKcs to DNA after the dsDNA cellulose pull-down assay (Fig. 2B). Although it was marginal, the addition of increasing amounts of target peptide (HNI-38), not control peptide (HN-26), led to a decrease in DNA-PKcs associated with dsDNA, suggesting that target peptide binds to DNA-PKcs and inhibits its binding to Ku70/Ku80. It is also noted that the addition of target peptide affected the binding of Ku70/Ku80 to the dsDNA cellulose (Fig. 2B). To further examine the effect of HNI-38 on the DNA binding activity of Ku, target peptide was incubated with purified Ku70/Ku80 complex in the presence of dsDNA cellulose, and the reaction mixtures were analyzed for the presence of Ku70 and Ku80 after the dsDNA pull-down assay (Fig. 2C). In keeping with Fig. 2B, target peptide (HNI-38) significantly interfered with binding of Ku complex to dsDNA under the conditions where control peptide (HN-26) showed virtually no effect (Fig. 2C). This result suggests that target peptide not only affects the interaction between DNA-PKcs and Ku complex, but also interferes with the DNA binding activity of Ku.

Effect of Target Peptide (HNI-38) on DNA-PK Kinase Activity. Interaction of DNA-PKcs with Ku complex is necessary for activation of its kinase activity (Gottlieb and Jackson, 1993; Hartley et al., 1995); therefore, the efficacy of target peptide was analyzed by measuring DNA-PK kinase activity in vitro in the presence of either HN-26 or HNI-38. DNA-PK kinase activity was inhibited up to 50% in the presence of HNI-38 under the conditions where a control peptide (HN-26) showed minimal effect (Fig. 3), strongly supporting the notion that target peptide specifically binds to DNA-PKcs and interferes with interaction between DNA-PKcs and Ku complex. Inhibitory effect of target peptide on DNA-PK occurred at low peptide concentrations (<20 nM) and, in the presence of 20 nM or higher, both target and control peptides inhibited DNA-PK activity (data not shown).

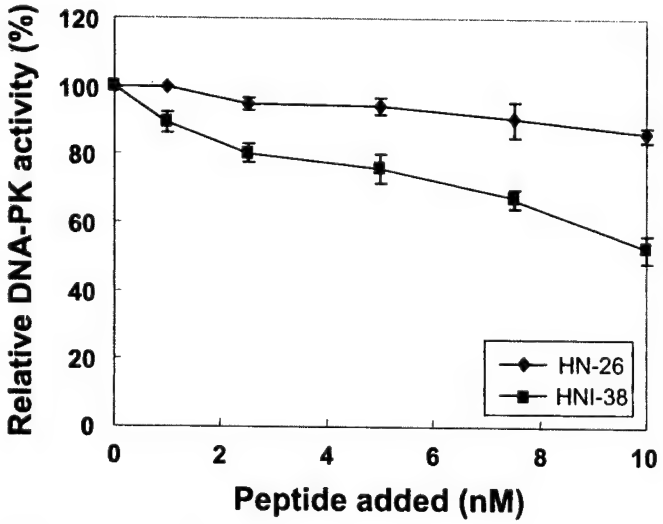


Fig. 3. Effect of target peptide on DNA-PK kinase activity in vitro. Partially purified DNA-PK fraction was incubated with various concentrations of either control peptide or target peptide prior to the addition of substrate peptide and other components for DNA-PK kinase assay (see *Materials and Methods* for details). DNA-PK activity was measured as the relative amounts of ^{32}P transferred to the substrate peptide.

Target Peptide Interferes with Repair of Double-Stranded DNA Breaks Induced by IR. IR-induced double-stranded DNA breaks are efficiently repaired by an NHEJ process. Genetic and biochemical studies strongly indicated that DNA-PK plays an essential role in NHEJ (Blunt et al., 1995; Jin et al., 1997; Jeggo, 1998). Hence, an alternative way to determine the efficacy of peptide inhibitor is to measure the repair of double-stranded DNA breaks following IR. Breast cancer cells (NCI) grown in the presence of [^{14}C]thymidine (DiBiase et al., 2000) were treated with either a control or a target peptide for 24 h. After irradiation (40 Gy), cells were harvested at various time points, and intact chromosomal DNA and DSBs were separated by pulsed field gel electrophoresis (0.5% agarose). Treatment of NCI cells with IR (40 Gy) induced substantial amounts of dsDNA breaks, most of which were repaired within 4 h. Cells treated with target or control peptide did not show any difference in generating DSBs after IR (Fig. 4A; lane 2 versus lanes 8 and 14). On the other hand, cells treated with target peptide (Fig. 4A, lanes 14–18) compared with those treated with control peptide (Fig. 4A, lanes 8–12) showed a noticeable decrease in DSB repair activity. This result suggests that target peptide interfered with dsb repair in vivo through the targeted inhibition of DNA-PK activity.

Target Peptide Inhibits Breast Cancer Cell Growth Only in the Presence of DNA Damage. Cells lacking DNA-PK catalytic subunit showed increased sensitivity to

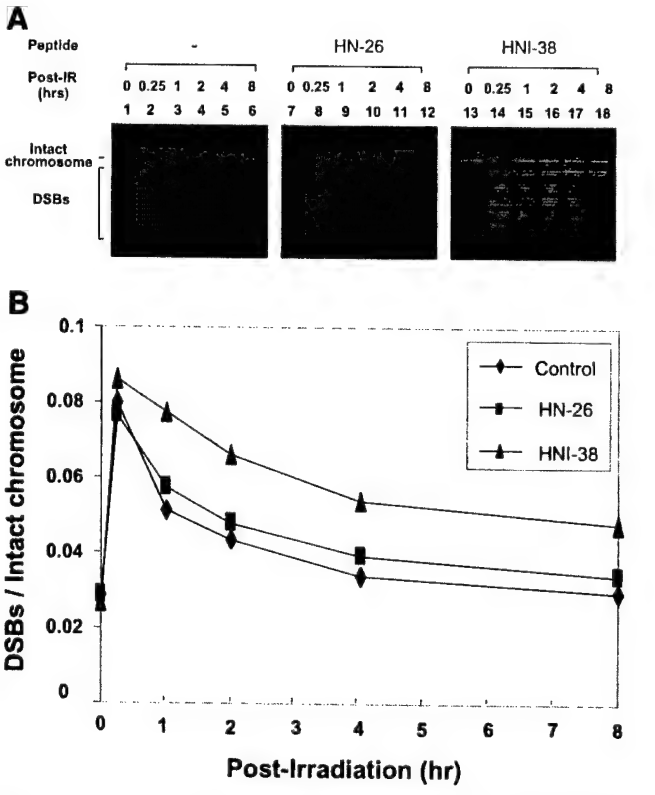


Fig. 4. Effect of target peptide on double-stranded DNA break repair. Breast cancer cells (NCI) grown in ^{14}C-containing media were treated with ionizing radiation (40 Gy) in the presence of no peptide (lanes 1–6), 50 nM control peptide (lanes 7–12), or 50 nM target peptide (lanes 13–18). After harvesting the cells at various time points, intact chromosomes and double-stranded DNA breaks (DSBs) were separated by gel electrophoresis (panel A, fluorography) and were quantified by liquid scintillation counter (panel B).

DNA-damaging drugs or IR (Kirchgessner et al., 1995; Lees-Miller et al., 1995), suggesting that DNA-PK activity is essential for DNA repair and cell survival upon DNA damage. We therefore tested whether a targeted inhibition of DNA-PK by a peptide, HNI-38, would sensitize breast cancer cells upon treatment of ionizing radiation or chemotherapeutic drug (cisplatin). Two breast cancer cell lines (NCI and MDA231) were treated with either control (HN-26) or target peptide (HNI-38) and tested for the efficacy of DNA-PK inhibitory peptide on lowering resistance of cells in response to ionizing radiation using a standard colony count cell survival assay. Neither control nor target peptides showed any effect on cell growth in the absence of ionizing radiation. However, cells treated with IR showed significant cell growth inhibition in the presence of target peptide but not with control peptide (Fig. 5A), suggesting that cell growth inhibition by target peptide occurs through targeting DNA-PK activity. Cells treated with cisplatin, although not as effective as those treated with ionizing radiation, also showed inhibitory effect on cell growth in the presence of HNI-38 (Fig. 5B).

Discussion

Many key human DNA repair pathways, such as double-strand break repair or nucleotide excision repair pathway, rely on multimeric polypeptide activities (Friedberg, 1996; Sancar, 1996; Wood, 1996; Lee, 2001). Interactions between damage recognition proteins and those proteins that report

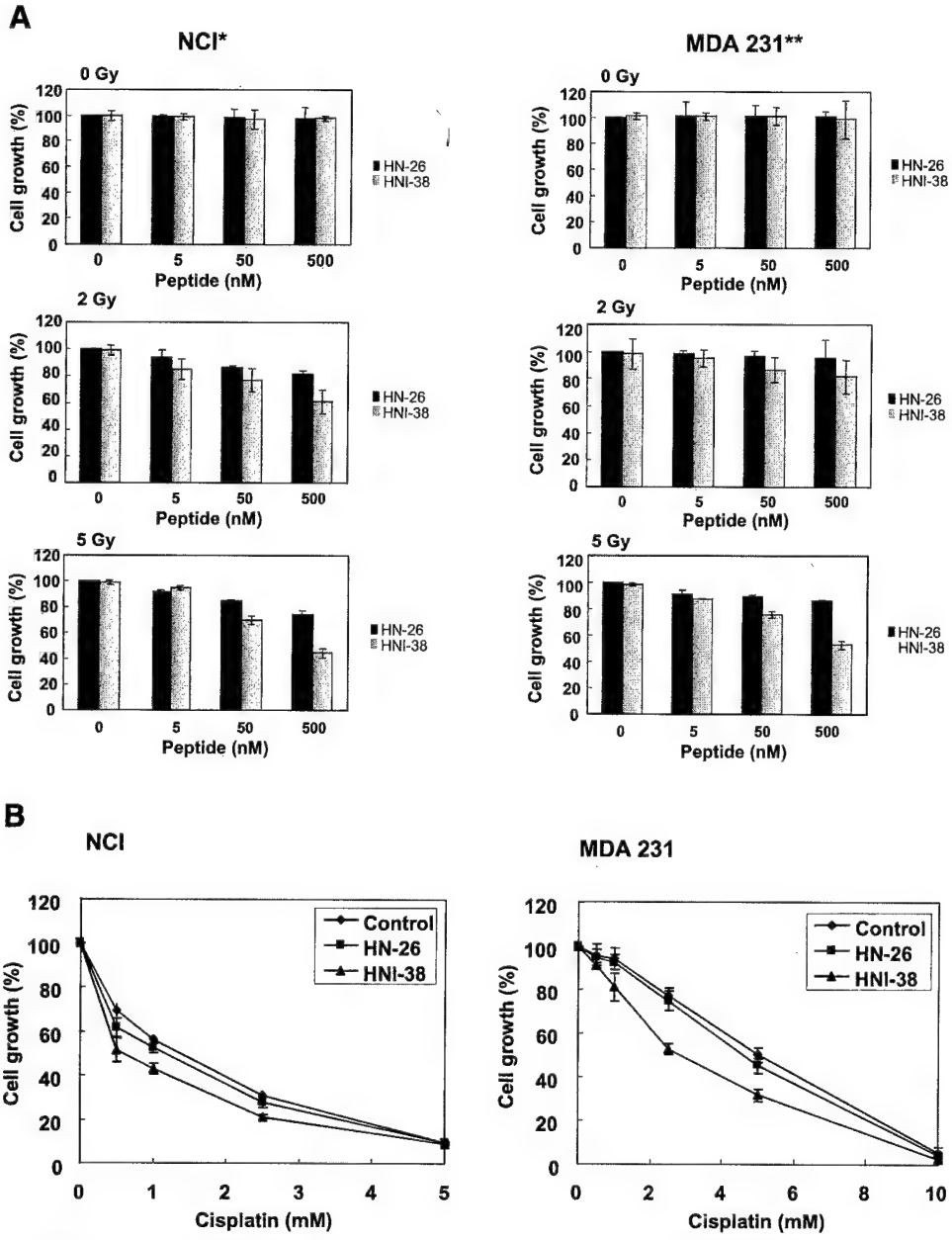


Fig. 5. Effect of control (HN-26) and target (HNI-38) peptides on the growth of breast cancer cells treated with ionizing radiation (panel A) or cisplatin (panel B). Values expressed are means (±S.E.) of the three replications (⋆, $p < 0.01$; ⋆⋆, $p < 0.01$). The clonogenic assay was used for the cells treated with ionizing radiation, and the cell survival assay (MTT) was used for those treated with cisplatin (see *Materials and Methods* for the detailed procedure).

the damage to downstream repair activities are crucial for DNA repair. DNA-PK is a key component of the NHEJ pathway with the unique property of being activated by double-stranded DNA breaks (Blunt et al., 1995). Earlier studies with drug-resistant and -sensitive cancer cells suggested that high level expression of DNA-PK leads to drug-resistant cells, whereas low DNA-PK activity was associated with drug-sensitive phenotype (Muller and Salles, 1997; Shen et al., 1997, 1998; Muller et al., 1998; Tew et al., 1998; Frit et al., 1999; Kim et al., 1999, 2000), implicating a role for DNA-PK in conferring cells becoming drug resistant in response to anticancer DNA-damaging drug. Since the interaction of DNA-PKcs to its regulatory subunits, Ku70 and Ku80, is crucial for its function in DNA repair, a targeted inhibition of DNA-PK would sensitize drug resistance of cancer cells and facilitate cell killing. Therefore, we attempted to develop a peptide cotherapy strategy in which a low molecular weight peptide-based inhibitor specifically interferes with interaction between DNA-PKcs and Ku complex.

A target peptide (HNI-38) containing the C terminus of Ku80 interfered with the interaction between DNA-PKcs and Ku complex. This was a much anticipated result since the C terminus of Ku80 was previously identified as DNA-PKcs interacting domain (Gell and Jackson, 1999). Inhibitory effect of HNI-38 on the interaction between DNA-PKcs and Ku70/Ku80 directly affected its kinase activity, showing inhibition of DNA-PK activity up to 50% under the conditions where a control peptide (HN-26) showed very little effect (Fig. 3). However, addition of an excess amount of target peptide did not show any further inhibition of DNA-PK kinase activity (data not shown). This is likely due to the fact that DNA-PKcs without Ku complex can still function as a kinase, although its activity is low. A target peptide (HNI-38) not only inhibited the interaction of DNA-PKcs with Ku complex on dsDNA, but also affected the dsDNA binding activity of Ku (Fig. 2C). It is not clear how HNI-38 interferes with DNA binding activity of the Ku complex; however, the C terminus of both Ku70 and Ku80 has been shown to be important for heterodimer assembly as well as for DNA-termini binding (Wu and Lieber, 1996; Gell and Jackson, 1999). It is possible that HNI-38 may interfere with the Ku70-Ku80 interaction through its binding to Ku70, which would negatively influence the DNA-termini binding activity of Ku.

DNA-PK activity is essential for DNA repair as well as cell cycle arrest in response to DNA damage, which contributes to cell survival by protecting cells from apoptosis. Cells treated with target peptide but not control peptide showed a noticeable decrease in DSB repair after a high dose (40 Gy) of IR, suggesting that HNI-38 specifically targets DNA-PK in vivo and interferes with dsb repair activity through inhibition of DNA-PK activity. Targeted inhibition of DNA-PK by HNI-38 also caused cell growth inhibition only when cells were treated with IR, suggesting that HNI-38 targeted DNA-PK and lowered resistance of cells in response to ionizing radiation, which eventually causes growth inhibition of both NCI and MDA231. Treatment of cells with HNI-38 also showed additive effect on cell growth inhibition in response to cisplatin treatment. This observation is in keeping with previous findings that DNA-PK is directly involved in nucleotide excision repair action in mammals (Muller et al., 1998). It also supports the notion that a targeted inhibition of DNA-PK would sensitize cancer cells upon treatment of chemotherapeutic drugs such as cisplatin. Taken together, our study results described here not only validate DNA-PK as a useful molecular target for the treatment of drug-resistant cancer cells, but also support a physiological role for DNA-PK in IR or chemotherapy drug resistance of cancer cells.

Acknowledgments

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Biochemical Analysis of the Damage Recognition Process in Nucleotide Excision Repair*

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XPA, XPC-hHR23B, RPA, and TFIIH all are the damage recognition proteins essential for the early stage of nucleotide excision repair. Nonetheless, it is not clear how these proteins work together at the damaged DNA site. To get insight into the molecular mechanism of damage recognition, we carried out a comprehensive analysis on the interaction between damage recognition proteins and their assembly on damaged DNA. XPC physically interacted with XPA, but failed to stabilize the XPA-damaged DNA complex. Instead, XPC-hHR23B was effectively displaced from the damaged DNA by the combined action of RPA and XPA. A mutant RPA lacking the XPA interaction domain failed to displace XPC-hHR23B from damaged DNA, suggesting that XPA and RPA cooperate with each other to destabilize the XPC-hHR23B-damaged DNA complex. Interestingly, the presence of hHR23B significantly increased RPA/XPA-mediated displacement of XPC from damaged DNA, suggesting that hHR23B may modulate the binding of XPC to damaged DNA. Together, our results suggest that damage recognition occurs in a multistep process such that XPC-hHR23B initiates damage recognition, which was replaced by combined action of XPA and RPA. XPA and RPA, once forming a complex at the damage site, would likely work with TFIIH, XPG, and ERCC1-XPF for dual incision.

Nucleotide excision repair (NER)[1] is one of the major repair pathways for removal of DNA damage caused by UV irradiation and a wide variety of bulky helix-distorting lesions such as cisplatin (1–4). In mammals, NER requires over 20 polypeptides, including damage recognition and/or structure distortion factors (XPA, XPC-hHR23B, replication protein A (RPA), and a transcription factor, TFIIH), strand separating helicases to create an open preincision complex (TFIIH containing XPB and XPD DNA helicases), two structure-specific endonucleases (ERCC1-XPF and XPG), and the enzymes needed for gap filling (DNA polymerase δ/ϵ, proliferating cell nuclear antigen, replication factor C, and RPA).

Both RPA and XPA are also known as damage recognition proteins because they preferentially bind to cisplatin- or UV-damaged DNA (5–10). Both proteins may also play a role in subsequent steps in NER through interaction with other repair proteins (8, 11–15). The XPA-DNA interaction is relatively weak and characterized by rapid dissociation, whereas RPA formed a much more stable complex with UV-damaged DNA (16). XPA physically interacts with RPA, which is necessary for efficient NER action (14). Wild-type RPA, but not a mutant lacking the XPA interaction domain, led to stabilization of XPA-damaged DNA complex, implicating a unique role for RPA in stabilizing the XPA-damaged DNA complex. The XPA-damaged DNA interaction is also likely necessary for recruiting other DNA repair proteins such as XPG, ERCC1-XPF, and TFIIH to the damaged site (8, 15, 17). RPA may also be involved in the later stage of NER, gap-filling, that requires proliferating cell nuclear antigen, replication factor C, and DNA polymerase δ (or ϵ) (18).

XPC-hHR23B is a human homolog of yeast Rad4 and Rad23 proteins, respectively, and forms a stable complex in solution. XPC-hHR23B exhibits the strongest affinity for damaged DNA (19–21), as does the yeast counterpart, Rad4-Rad23 (22). Rad23 without Rad4 does not show any DNA binding activity, suggesting that Rad4 is solely responsible for recognition of damaged DNA. Rad23 is essential for XPC function in NER and may also be necessary for the solubility of Rad4 (23). XPC-hHR23B showed a remarkable preference to UV-damaged DNA particularly in the presence of nondamaged competitor DNA and has been suggested as the initiator of global genome NER (19, 24). A recent immunohistochemistry study also strongly supports a role for XPC as a global initiator in repair (25), while suggesting a role for XPA and RPA as repair mediator proteins. XPC-hHR23B is also involved in the recruitment of TFIIH to damaged DNA (26). TFIIH, once recruited, may play a role in distinguishing the damaged strand from the nondamaged one (27) as well as local unwinding of the damaged DNA region. TFIIH with its DNA helicase activity likely generates a junction between single-stranded DNA and duplex DNA that is recognized by two structure-specific endonucleases, XPG and ERCC1-XPF, for dual incision of damaged strand.

An ongoing challenge is to understand how DNA damage is recognized and distinguished from nondamaged sites. In mammalian cells, XPC-hHR23B, XPA, RPA, and TFIIH factors may all have roles in damage recognition during the early stage of NER. In this study we carried out a comprehensive analysis on the interaction between damage recognition proteins and their assembly on damaged DNA. We found that XPC-hHR23B, like

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[1] The abbreviations used are: NER, nucleotide excision repair; ERCC, excision repair cross-complementing; GST, glutathione *S*-transferase; hHR23, human homolog of the yeast RAD23; ITR-60, intrastrand cross-linked duplex DNA of 60-mer; RPA, replication protein A; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; TFIIH, transcription factor IIH; XP, xeroderma pigmentosum; TET, tetrachlorofluorescein.

RPA, physically interacted with XPA. However, the XPA-XPC interaction, unlike the XPA-RPA interaction, failed to stabilize the XPA-damaged DNA complex. Instead, XPA cooperates with RPA to promote the destabilization of the XPC-hHR23B-damaged DNA interaction. This finding supports a notion that the damage recognition process occurs in a stepwise manner such that XPC-hHR23B initiates damage recognition, which was replaced by the combined action of XPA and RPA.

MATERIALS AND METHODS

Preparation of Platinum-induced Damaged DNA—To study the interaction between damaged DNA and damage recognition factors, we constructed a duplex DNA with the cisplatin lesion at a specific site (Fig. 1*A*). Oligonucleotides containing an intrastrand (ITR-60) cross-link were prepared according to the previously described procedure with some modifications (28). For cross-linking, the top strand was first incubated with cisplatin (in TE, pH 8.0, 2-fold molar excess) at 37 °C in the dark for 48 h, and then ethanol-precipitated. The damaged DNA was purified by 15% denaturing polyacrylamide gel electrophoresis and annealed to the bottom strand (5-fold molar excess). The duplex DNA was purified by 15% native polyacrylamide gel electrophoresis. Purified duplex DNA, 5′-^{32}P-labeled at top strand, was analyzed for intrastrand cross-link by treatment with 10% dimethyl sulfate at room temperature for 5 min and 1 M piperidine at 90 °C for 30 min, followed by 15% denaturing polyacrylamide gel electrophoresis (Fig. 1*B*).

Purification of XPA, RPA, and XPC-hHR23B—Both RPA and XPC-hHR23B are multiprotein complexes that can be highly expressed in insect cells by coinfecting recombinant baculoviruses (21, 29). RPA complex was prepared by coinfecting recombinant baculoviruses encoding the individual subunits (29) and the XPC-hHR23B complex was from insect cell lysates infecting a recombinant baculovirus containing both XPC and hHR23B genes (Ref. 21; recombinant baculovirus containing XPC and hHR23B was kindly provided by Dr. A. Sancar, University of North Carolina, Chapel Hill, NC). A high level of histidine-tagged XPA was expressed in *Escherichia coli* under control of the T7 promoter (5). Both RPA and XPA have been isolated with more than 95% purity using a series of conventional column chromatography as described previously (5, 13, 29) (Fig. 1*C*). XPC-hHR23B complex was purified using a series of column chromatography (UV-damaged dsDNA cellulose, phosphocellulose (P11), and hepharin-Sepharose) (Fig. 1*C*, the details of XPC-hHR23B purification will be described elsewhere). All three proteins (XPA, RPA, and XPC-hHR23B) were functionally active in supporting *in vitro* NER activity with HeLa cell extracts lacking respective protein (data not shown). All GST fusion proteins were purified using glutathione-Sepharose affinity column as described previously (14). TFIIH was isolated from HeLa cell nuclear extracts using an antibody affinity procedure described previously (31). During purification, TFIIH was monitored by Western blot using anti-XPB and -XPD antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

GST Fusion Pull-down Assay—The indicated amount of GST fusion protein was incubated with glutathione-Sepharose beads (25 μl) in 0.5% nonfat milk at 4 °C for 30 min, and washed three times with washing buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.5% Nonidet P-40, 150 mM NaCl, 200 μg/ml bovine serum albumin). After washing the beads 3–5 times, beads were incubated with the indicated protein either in the presence or absence of damaged DNA for an additional 20 min at room temperature on a rocker and then washed 5 times with washing buffer. Beads were then mixed with 25 μl of SDS-PAGE sample buffer, heated at 95 °C for 5 min, and proteins (or DNA) were resolved on 10% SDS-PAGE. To quantitatively measure the damaged DNA, the gel was directly scanned on a Hitachi FMBIO II Fluorescent Image Scanner.

SDS-PAGE and Western Blot Analysis—Protein samples were separated by 10% SDS-PAGE as described previously (32). Proteins were then transferred to polyvinylidene difluoride membranes, immunoblotted with corresponding antibodies, and detected by ECL plus Western blotting detection reagents (Amersham Biosciences).

Protein-damaged DNA Interaction: Surface Plasmon Resonance Analysis—Interactions of XPA, RPA, and XPC-hHR23B with DNA were monitored using a surface plasmon resonance biosensor instrument, Biacore 3000 (Biacore) as described previously (16, 34). For preparation of the biosensor surface with DNA, 5′-biotinylated 60-mer duplex DNA (prepared in a buffer containing 10 mM sodium acetate, pH 4.8, and 1.0 M NaCl) was manually injected onto a streptavidin-coated surface of a BIAcore sensor chip to the desired density in different flow cells. One flow cell was left underivatized to allow for refractive index change correction. Proteins were diluted in the running buffer containing 10 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 0.005% polysorbate-20, and 1 mM dithiothreitol. Each experiment was repeated at least twice to assure reproducibility.

RESULTS

Interaction of Damage Recognition Factors with DNA—To get insight into the molecular mechanism of how damage recognition proteins are assembled and working together at the damaged DNA site, we carried out a comprehensive analysis on the interaction of XPA, RPA, and XPC-hHR23B with damaged DNA. Binding affinity of damage recognition factors to a duplex DNA containing an intrastrand platinum cross-link (ITR-60, Fig. 1) was measured using an electrophoretic mobility shift assay. As previously reported (Refs. 1–4 and references therein), both RPA and XPC-hHR23B showed preferential binding to the cisplatin-damaged DNA over the nondamaged one, whereas the XPA-DNA interaction was observed only in the presence of excess amounts (Fig. 2*A*). On the other hand, surface plasmon resonance kinetic analysis indicated that XPC-hHR23B compared with RPA exhibited much higher affinity to the damaged DNA (Fig. 2*B*). This finding is in keeping with the previous observation that XPC has a considerable preference for binding to the damaged DNA over nondamaged competitor DNA (24, 25). XPC-hHR23B also differs from RPA in its preferential binding to dsDNA over ssDNA (35), whereas RPA has a much higher affinity to ssDNA than to dsDNA (Fig. 2, *A* and *B*).

XPC Physically Interacts with XPA, but Not with RPA—Because all three proteins (RPA, XPA, and XPC-hHR23B) independently bind to the damaged DNA, we examined a possibility whether all three damage recognition proteins form a complex on the damaged DNA site (11–13). Initially, we examined the interactions among damage recognition factors without DNA. Because we know that XPA and RPA interact with each other in solution as well as on damaged DNA (14, 17), our focus was on the interaction of XPC-hHR23B with RPA or XPA (Fig. 3). GST·XPC or GST·XPC-hHR23B were incubated with an increasing amount of XPA (or RPA), pulled down with glutathione-Sepharose beads for co-precipitation of XPA (or RPA), and analyzed by Western blot (Fig. 3). XPA was co-precipitated with GST·XPC or GST·XPC-hHR23B complex, but not with GST (Fig. 3*A*), suggesting that XPA physically interacts with XPC-hHR23B. In the XPA-XPC interaction, GST·XPC was almost comparable with GST·XPC-hHR23B (Fig. 3A, *lanes 3–4 versus lanes 5* and *6*), indicating that XPC not hHR23B physically interacts with XPA. In keeping with this, hHR23B without XPC failed to interact with XPA (data not shown). Unlike XPA, however, RPA had no physical interaction with GST·XPC-hHR23B or GST·XPC in solution (Fig. 3*B*).

Because both RPA and XPC interact with XPA (Refs. 11–13 and Fig. 3), it is possible that the binding of XPC (or RPA) to XPA may competitively exclude RPA (or XPC) from the complex. If so, we may not be able to see a complex containing all three damage recognition proteins. To examine this possibility, XPA was incubated with GST·XPC-hHR23B in the presence of an increasing amount of RPA and analyzed for the XPA-XPC interaction. Addition of a molar excess of RPA had little or no effect on the XPA-XPC interaction as determined by the GST·XPC-hHR23B pull-down assay (Fig. 3*C*), suggesting that RPA and XPC-hHR23B may recognize two separate domains of XPA. Nonetheless, we were not able to detect RPA in the GST·XPC pull-down assay in the presence of XPA and RPA (Fig. 3*C*).

Presence of Damaged DNA Significantly Inhibits the XPA-XPC Interaction—The XPA-damaged DNA interaction is weak, but markedly stimulated in the presence of a high affinity DNA-binding protein, RPA (14). The stimulatory effect of RPA

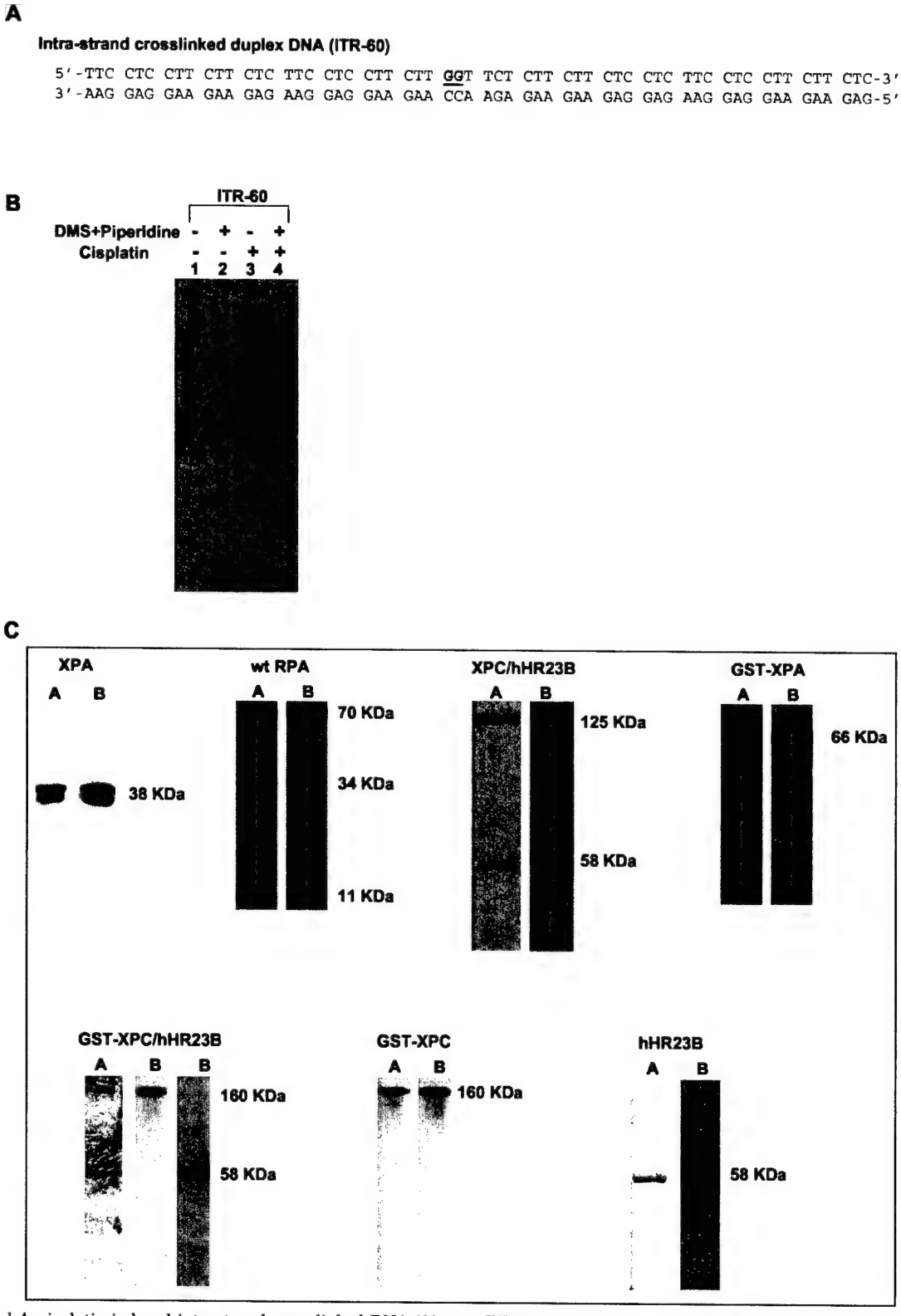


FIG. 1. *Panel A*, cisplatin-induced intrastrand cross-linked DNA (60-mer; ITR-60). *Underlined* guanine residues (*G*) are the reactive sites for platination. *Panel B*, the duplex DNA with intrastrand cross-link (ITR-60) showed slower mobility on a 15% denatured polyacrylamide gel electrophoresis (*lanes 1 versus 3*). The Maxam-Gilbert G-residue reaction (DMS + piperidine treatment) showed protection of ^{32}P-labeled DNA from cleavage in intrastrand cross-linked DNA (*lanes 2 versus 4*). Cisplatin-damaged duplex DNA was labeled at the 5′-end of the nondamage-containing strand with fluorescence dye (TET, synthesized from the Integrated DNA Technologies, Coralville, IA). *Panel C*, SDS-PAGE and Western blot analysis of purified XPA, RPA, and XPC and/or hHR23B. Individual proteins were electrophoretically separated by 12% SDS-PAGE and visualized by Coomassie Blue staining (*left lane*; see "Materials and Methods" for the details) as well as by immunoblot using the corresponding polyclonal antibody (*right lane*).

on the XPA-damaged DNA interaction occurs through the RPA-XPA interaction (14, 16). Similar to RPA, XPC-hHR23B exhibits a high affinity to damaged DNA (19–23) and physically interacts with XPA (Fig. 3). We therefore examined whether XPC-hHR23B affects the XPA-damaged DNA interaction. In the GST-XPA pull-down assay, XPA had very little or no bind-

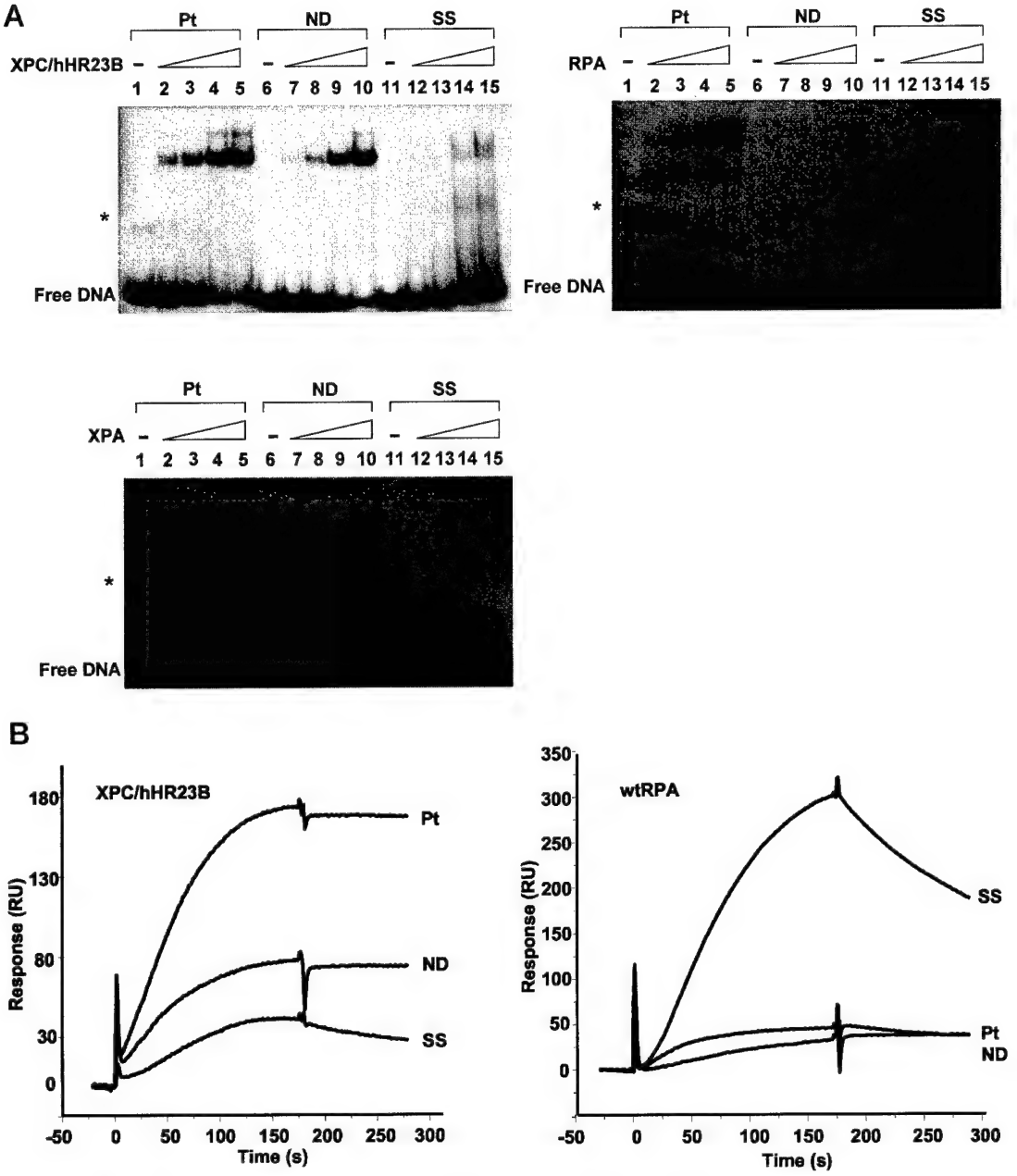


FIG. 2. **Binding characteristics of XPA, RPA, and XPC-hHR23B to the cisplatin-damaged DNA (ITR-60).** *Panel A*, increasing amounts of XPC-hHR23B (*top panel*; *lanes 1*, *6*, and *11*, 0 ng; *lanes 2*, *7*, and *12*, 20 ng; *lanes 3*, *8*, and *13*, 40 ng; *lanes 4*, *9*, and *14*, 60 ng; *lanes 5*, *10*, and *15*, 100 ng), RPA (*middle panel*; *lanes 1*, *6*, and *11*, 0 ng; *lanes 2*, *7*, and *12*, 10 ng; *lanes 3*, *8*, and *13*, 20 ng; *lanes 4*, *9*, and *14*, 40 ng; *lanes 5*, *10*, and *15*, 80 ng), or XPA (*bottom panel*; *lanes 1*, *6*, and *11*, 0 ng; *lanes 2*, *7*, and *12*, 35 ng; *lanes 3*, *8*, and *13*, 70 ng; *lanes 4*, *9*, and *14*, 175 ng; *lanes 5*, *10*, and *15*, 350 ng) was incubated with 100 fmol of 5′-^{32}P-damaged duplex DNA (ITR-60; *lanes 1–5*), 5′-^{32}P-nondamaged duplex DNA (*lanes 6–10*), or 5′-^{32}P-ssDNA (*lanes 11–15*) for 15 min at room temperature. The protein-DNA complex was analyzed by 4% polyacrylamide gel in 0.5× TBE (acrylamide:bisacrylamide = 43.2:0.8). For quantification, regions corresponding to protein-DNA complex were excised and measured for radioactivity. *Asterisk* indicates the ssDNA fragment (60-mer) generated from ITR-60 during gel purification. *Panel B*, biomolecular interaction (BIAcore) analysis of the interaction of XPC-hHR23B and RPA with damaged DNA (ITR-60). XPC-hHR23B or RPA (25 nM) was injected into ssDNA (SS), nondamaged duplex DNA (ND), or cisplatin-damaged DNA (platinum; *Pt*) surface (50 response units) using the KINJECT function of BIAcore 3000. The association phase was allowed for 180 s followed by a 120-s buffer injection period for dissociation (see "Materials and Methods" for details).

ing to the damaged DNA (Fig. 4*A*, *lane 2*), which was significantly stimulated by addition of RPA (Fig. 4*A*, *lanes 3–5*). In contrast, XPC-hHR23B showed no effect on the XPA-damaged DNA interaction (Fig. 4*A*, *lanes 6–8*). Interestingly, the stimulatory effect of RPA on the XPA-damaged DNA interaction was markedly reduced in the presence of XPC-hHR23B (Fig. 4*A*, *lane 9*), suggesting that XPC-hHR23B, unlike RPA, may not form a stable complex with XPA on damaged DNA. To investigate this further, we examined the effect of damaged DNA on the XPA-XPC interaction (Fig. 4*B*). XPA was successfully co-precipitated with GST·XPC in the pull-down assay in the absence of damaged DNA (Fig. 4*B*, *lanes 4* and *5*). In the presence of damaged DNA, however, the amount of XPA co-precipitated with GST·XPC was significantly reduced to an undetectable level (Fig. 4*B*, *lanes 6* and *7*), suggesting that the XPA-XPC interaction is destabilized in the presence of damaged DNA.

XPC-hHR23B Is Displaced from Damaged DNA by the Combined Action of RPA and XPA—Our result (Fig. 4) strongly suggests that XPA, RPA, and XPC-hHR23B do not form a

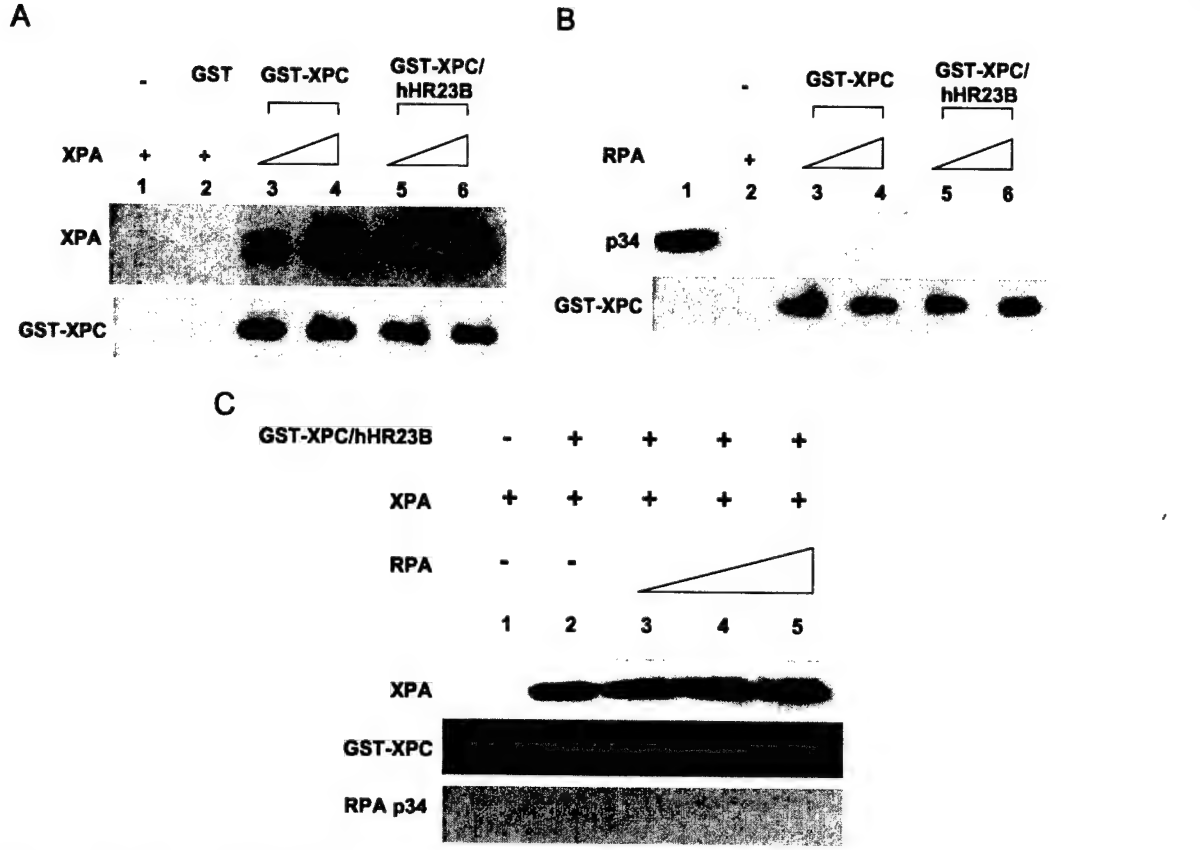


FIG. 3. **XPC interacts with XPA, but not with RPA.** *Panel A*, 2 pmol of GST-XPC or GST-XPC-hHR23B was mixed with glutathione-Sepharose beads (25 μl) and gently rocked in the presence of XPA (2 pmol (*lanes 3* and *5*) and 5 pmol (*lanes 4* and *6*)) at room temperature for 20 min. After incubation, beads were washed with buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.5% Nonidet P-40, 150 mM NaCl, 200 μg/ml bovine serum albumin), and the bound XPA was analyzed by Western blot using an anti-XPA polyclonal antibody. *Panel B*, GST-XPC or GST-XPC-hHR23B (2 pmol) was mixed with glutathione beads (25 μl) in the presence of RPA (2 pmol (*lanes 3* and *5*) or 5 pmol (*lanes 4* and *6*)) at room temperature for 20 min. After incubation, beads were washed with buffer and bound RPA was analyzed by Western blot using an anti-RPA p34 polyclonal antibody. *Lane 1* contained purified RPA as a control.

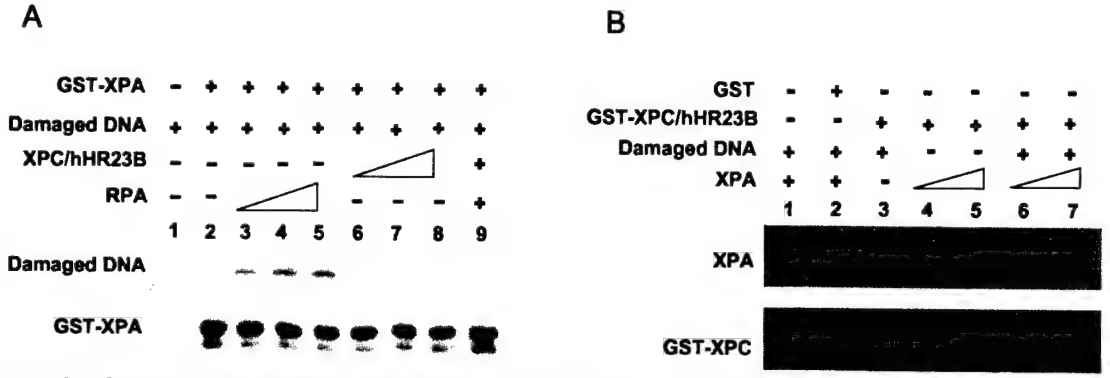


FIG. 4. **Interaction between XPC-hHR23B and XPA on damaged DNA.** *Panel A*, RPA not XPC stabilizes the XPA-damaged DNA interaction. GST-XPA (2 pmol) was incubated with 2 pmol of 5′-TET-labeled cisplatin-damaged DNA (60-mer) in the presence of increasing amounts of RPA (2, 5, and 10 pmol in *lanes 3–5*, respectively) or XPC-hHR23B (2, 5, and 10 pmol in *lanes 6–8*, respectively). Following the GST pull-down assay (as described in the legend to Fig. 3*A*), precipitates were separated by 10% SDS-PAGE and analyzed for TET-labeled damaged DNA using a fluorescent image scanner (Hitachi FMBIO II) (*top panel*) or GST-XPA using an anti-GST antibody (*bottom panel*). *Panel B*, the presence of damaged DNA significantly inhibits the XPA-XPC interaction. Two pmol of GST (*lane 2*) or GST-XPC-hHR23B (*lanes 3–7*) was incubated with increasing amount of XPA (2 pmol in *lanes 2*, *4*, and *7*; 5 pmol in *lanes 5* and *7*) for 20 min at room temperature. Where indicated, 2 pmol of cisplatin-damaged DNA (ITR-60) was included. Following the GST pull-down, precipitates were separated (10% SDS-PAGE) and analyzed by Western blot using an anti-XPA (*top panel*) or -GST (*bottom panel*) antibody.

stable complex on the damaged DNA. Instead, the damage recognition process may occur in a stepwise manner such that XPC-hHR23B interacts with damaged DNA first and then is replaced by XPA and/or RPA. To test this, biotin-labeled cisplatin-damaged DNA was first incubated with XPC-hHR23B, followed by the addition of RPA (Fig. 5*A*, *lanes 3–5*), XPA (*lanes 6–8*), or XPA + RPA (*lanes 9–11*) to the reaction mixtures. The streptavidin-Sepharose pull-down assay revealed that XPC-hHR23B formed a stable complex with damaged DNA (Fig. 5*A*, *top panel*, *lane 2*) and, the addition of RPA (*lanes 3–5*) or XPA (*lanes 6–8*) showed very little effect on the XPC-hHR23B-damaged DNA interaction. In the presence of both XPA and RPA, however, the XPC-hHR23B-damaged DNA interaction was significantly inhibited (Fig. 5*A*, *top panel*, *lanes 9–11*). In contrast to XPC, the amount of XPA co-precipitated with damaged DNA was markedly increased in the presence of RPA (Fig. 5*A*, *second panel*, *lanes 9–11*), whereas the RPA-damaged DNA interaction was hardly affected by the presence of XPA (Fig.

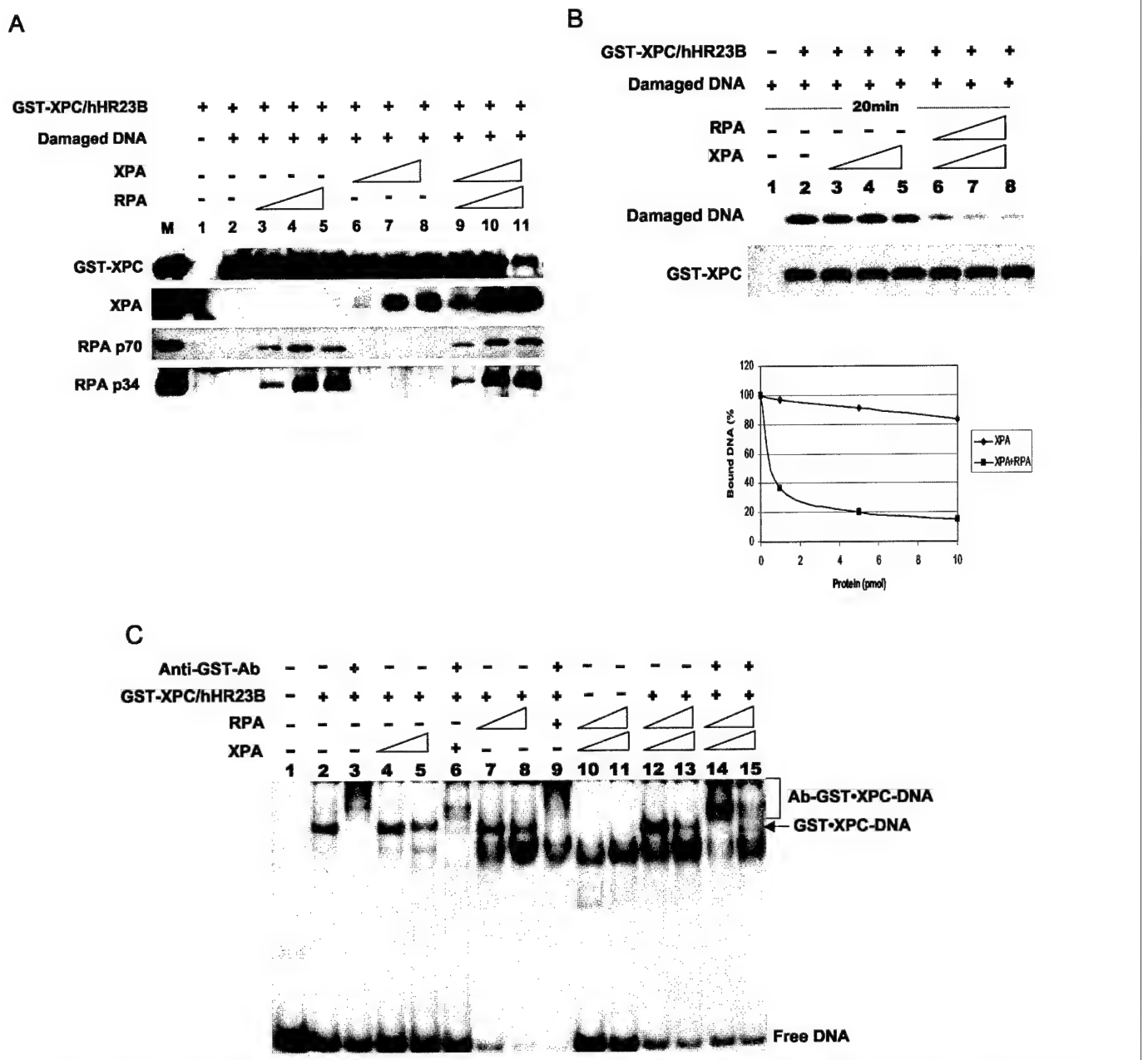


FIG. 5. **XPC-hHR23B is displaced from the damaged DNA by a combined action of XPA and RPA.** *Panel A*, Western blot analysis of individual damage recognition factors associated with biotin-labeled damaged DNA. Biotin-labeled damaged DNA (ITR-60; 2 pmol) was preincubated with streptavidin-Sepharose prior to the addition of GST·XPC-hHR23B (800 ng in *lanes 1–11*), RPA (2, 5, and 10 pmol in *lanes 3–5*, respectively), XPA (2, 5, and 10 pmol in *lanes 6–8*, respectively), and XPA + RPA (2, 5, and 10 pmol each in *lanes 9–11*, respectively). Following the pull-down of beads, proteins were analyzed by Western blot using an anti-GST (for GST·XPC), -XPA, -RPAp70, or RPAp34 antibody. In *lane M*, purified protein (GST·XPC, XPA, or RPA for p70 and p34) was included. *Panel B*, glutathione-Sepharose beads (25 μl) were mixed with GST·XPC-hHR23B (400 ng) and fluorescence (TET)-labeled damaged DNA (2 pmol), and gently rocked for 20 min at room temperature. After challenging the complex with either XPA (2, 5, and 10 pmol in *lanes 3–5*, respectively) or XPA + RPA (2, 5, and 10 pmol each in *lanes 6–8*, respectively), beads were pulled down and analyzed by 10% SDS-PAGE for TET-labeled damaged DNA using fluorescence image scanner (*top panel*) or Western blot using an anti-GST antibody (*bottom panel*). Relative amounts of TET-labeled damaged DNA are indicated at the *bottom* of the figure. *Panel C*, effect of RPA and XPA on the interaction between XPC and damaged DNA in a gel mobility shift assay. GST·XPC-hHR23B (20 ng) was incubated with 100 fmol of ^{32}P-platinum-damaged DNA (ITR-60) and incubated for 20 min at room temperature. Where indicated, XPA (20 ng in *lanes 4*, *10*, *12*, and *14*; 50 ng in *lanes 5*, *11*, *13*, and *15*), RPA (20 ng in *lanes 7*, *10*, *12*, and *14*; 50 ng in *lanes 8*, *11*, *13*, and *15*), or an anti-GST antibody (5 μl; 100 μg/ml) was added to the mixtures and further incubated for 20 min at room temperature prior to gel electrophoresis. The protein-DNA complex was analyzed by 4% polyacrylamide gel in 0.5× TBE (acrylamide:bisacrylamide = 43.2:0.8). GST/XPC-damaged DNA complex and its supershifted complexes are indicated by *arrows*. No protein was included in *lane 1*.

5A, *third* and *fourth panels*, *lanes 9–11*). Together, this result suggests that XPC-hHR23B can be effectively displaced from damaged DNA by the combined action of RPA and XPA.

We further analyzed the effect of XPA and/or RPA on the interaction between XPC-hHR23B and damaged DNA by measuring damaged DNA co-precipitated with GST·XPC-hHR23B in the glutathione-Sepharose pull-down assay. Fluorescence (TET)-labeled damaged DNA was first incubated with GST·XPC-hHR23B before the addition of either XPA or XPA + RPA and analyzed quantitatively following GST·XPC pull-down assay (Fig. 5*B*). Addition of both XPA and RPA drastically reduced the amount of damaged DNA interacting with GST·XPC-hHR23B, whereas XPA alone marginally affected the XPC-damaged DNA interaction (Fig. 5*B*), suggesting that

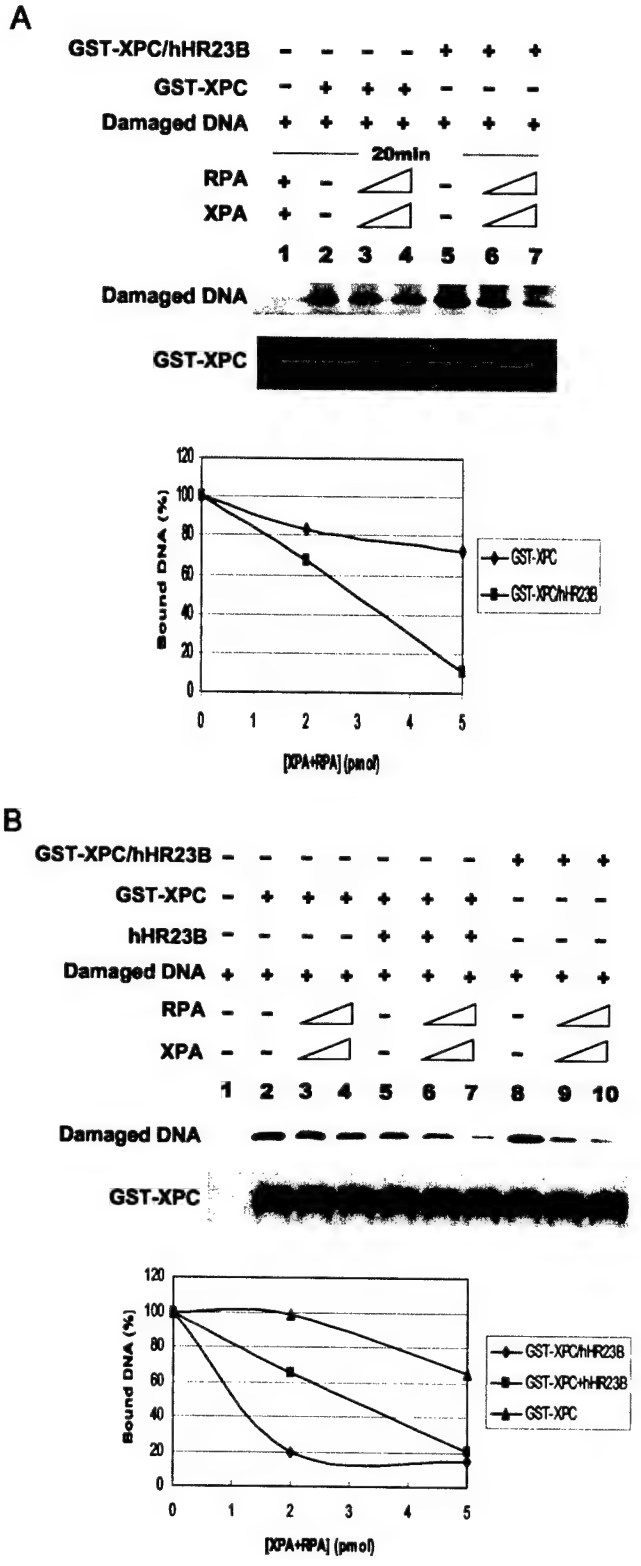


FIG. 6. **hHR23B is necessary for the displacement of XPC from damaged DNA.** *Panel A*, XPC-hHR23B not XPC is effectively displaced from damaged DNA. Two pmol of either GST·XPC (*lanes 2–4*) or GST·XPC-hHR23B (*lanes 5–7*) was mixed with glutathione-Sepharose beads (25 μl) and TET-labeled cisplatin-damaged DNA (2 pmol) and rocked for 20 min at room temperature. After adding increasing amounts of RPA + XPA (2 pmol (*lanes 3* and *6*) or 5 pmol (*lanes 1*, *4*, and *7*)), beads were washed and analyzed for TET-labeled cisplatin-damaged DNA by 10% SDS-PAGE. Relative amounts of TET-labeled DNA are indicated at the *bottom* of the figure. *Panel B*, hHR23B stimulates XPA/RPA-mediated displacement of XPC from the damaged DNA. Two pmol of GST·XPC (*lanes 2–7*) or GST·XPC-hHR23B (*lanes 8–10*) was mixed with glutathione beads (25 μl) and TET-labeled damaged DNA (2 pmol), and where indicated, 2 pmol of purified hHR23B was included. After rocking for 20 min at room temperature, equimolar amounts of RPA + XPA (2 pmol (*lanes 3*, *6*, and *9*) or 5 pmol (*lanes 4*, *7*, and *10*)) were added to the mixtures, and beads were pulled down and analyzed for TET-labeled damaged DNA by 10% SDS-PAGE. Relative amounts of TET-labeled DNA are indicated at the *bottom* of the figure.

both RPA and XPA are necessary to destabilize the interaction between XPC-hHR23B and damaged DNA. Effect of XPA and/or RPA on the GST·XPC-hHR23B-damaged DNA interaction was also analyzed by an antibody supershift assay using an anti-GST antibody (Fig. 5*C*). On a native gel electrophoresis, GST·XPC-hHR23B-damaged DNA complex was identified as a distinct band (Fig. 5*C*, *lane 2*) that was supershifted in the presence of an anti-GST antibody (Fig. 5*C*, *lane 3*). The GST·XPC-hHR23B-damaged DNA complex (*lanes 12* and *13*) or its supershifted complex (*lanes 14* and *15*) was significantly reduced by addition of an increasing amount of both RPA and XPA, even though the supershift may be in part because of the reaction of GST antibody to XPA and/or RPA (Fig. 5*C*). This result is in keeping with Fig. 5, *A* and *B*, indicating that XPC-hHR23B is displaced from damaged DNA by the combined action of RPA and XPA.

hHR23B Is Necessary for XPA/RPA-mediated Displacement of XPC from Damaged DNA—XPC (Rad4) forms a stable complex with hHR23B (Rad23), which appears to be essential for NER (23). Nonetheless, damaged DNA binding activity of the XPC-hHR23B complex belongs to the XPC subunit (23) and the exact role for hHR23B in repair is unclear. In an effort to explore the role for hHR23B in DNA repair, we examined whether the presence of hHR23B affects RPA/XPA-mediated displacement of XPC from the damaged DNA. GST·XPC-hHR23B (or GST·XPC) was incubated with fluorescence (TET)-labeled damaged DNA in the presence of RPA and XPA, and the amount of damaged DNA precipitated with GST·XPC was measured (Fig. 6*A*). Both XPC and XPC-hHR23B showed strong interaction with damaged DNA (Fig. 6*A*, *lanes 2* and *5*). In the presence of RPA + XPA, however, the damaged DNA·XPC-hHR23B complex, not the damaged DNA·XPC complex, was significantly reduced (Fig. 6*A*, *lanes 3* and *4 versus 6* and *7*), suggesting that hHR23B is somehow involved in the displacement of XPC from damaged DNA in the presence of RPA + XPA. To further investigate this, purified hHR23B was added to the reaction mixtures containing GST·XPC for its effect on the XPC-damaged DNA interaction in the presence of RPA + XPA (Fig. 6*B*). GST·XPC without hHR23B formed a stable complex with damaged DNA (Fig. 6*B*, *lane 2*), which was hardly affected by RPA + XPA (Fig. 6*B*, *lanes 3* and *4*). In contrast, addition of purified hHR23B to the reaction mixtures significantly decreased the binding of XPC to the damaged DNA in the presence of RPA + XPA (Fig. 6*B*, *lanes 6* and *7*). This result strongly suggests that hHR23B is necessary for the RPA/XPA-mediated displacement of XPC from damaged DNA.

The RPA-XPA Interaction Is Essential for the Displacement of XPC-hHR23B from Damaged DNA—Interaction between RPA and XPA is not only required to stabilize the XPA-damaged DNA interaction, but also necessary for NER activity (14, 16). Because both RPA and XPA are essential for the displacement of XPC from damaged DNA, we examined whether the RPA-XPA interaction is necessary for it. For this, wild-type RPA and two RPA mutants (RPAp34C33 lacking the C terminus of p34 subunit (XPA interaction domain); ZFM4, a mutant with cysteine to alanine substitution at the zinc finger domain of the p70 subunit) were compared in the displacement of XPC-hHR23B from damaged DNA. Although both mutants poorly supported NER activity *in vitro* (14, 36), ZFM4 supported the displacement of XPC-hHR23B from damaged DNA, whereas RPAp34C33 did not (Fig. 7). This result not only

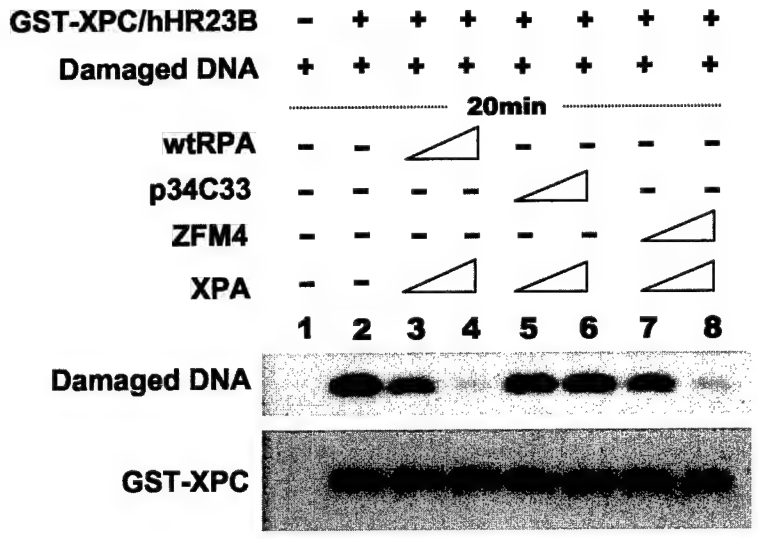
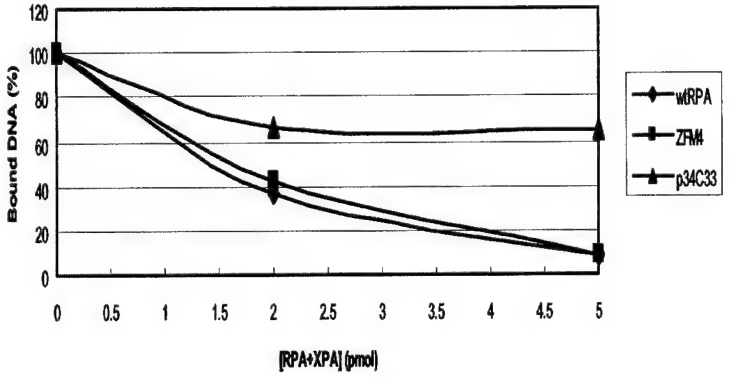


FIG. 7. **A mutant RPA lacking the XPA interaction domain did not support the displacement of XPC from damaged DNA.** GST·XPC-hHR23B (400 ng) was mixed with glutathione-Sepharose beads and TET-damaged DNA (2 pmol), and rocked for 20 min at room temperature. The mixtures were then challenged with increasing amounts of XPA (2 pmol (*lanes 3*, *5*, and *7*) or 5 pmol (*lanes 4*, *6*, and *8*)) in the presence of wild-type RPA (*lanes 3* and *4*), a mutant lacking C terminus of p34 (*lanes 5* and *6*), or a RPA zinc finger mutant (*ZFM4*) (*lanes 7* and *8*). Following the GST pull-down, beads were washed and analyzed for TET-labeled damaged DNA by 10% SDS-PAGE. Relative amounts of TET-labeled DNA are indicated at the *bottom* of the figure.



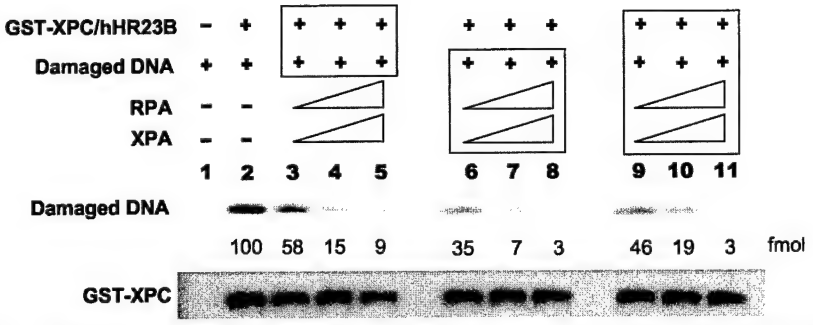


FIG. 8. **Displacement of XPC-hHR23B from damaged DNA is not affected by the order of assembly of damage recognition proteins.** The components indicated *inside* the *boxes* were incubated together for 20 min at room temperature prior to the addition of the remaining components. Where indicated, 400 ng of GST·XPC-hHR23B, 2 pmol of TET-labeled damaged DNA, and equimolar amounts of XPA + RPA (2 pmol (*lanes 3*, *6*, and *9*), 5 pmol (*lanes 4*, *7*, and *10*), or 10 pmol (*lanes 5*, *8*, and *11*)) were added. Following the GST pull-down, beads were washed and analyzed for TET-labeled damaged DNA by 10% SDS-PAGE. Relative amounts of TET-labeled DNA are indicated at the *bottom* of the figure.

suggests that RPA and XPA cooperate with each other in the displacement of XPC-hHR23B from damaged DNA, but also supports that the RPA-XPA interaction may be necessary for such cooperation.

Displacement of XPC-hHR23B from Damaged DNA Is Not Affected by the Order of Protein Assembly on Damaged DNA—Our finding that XPA and RPA cooperate with each other to displace XPC-hHR23B from the damaged DNA supports a notion that XPC-hHR23B is the initiator of global genome NER (19, 23, 24). To investigate this further, we examined whether the order of protein assembly at the damaged DNA site affects the XPC-hHR23B-damaged DNA interaction (Fig. 8). The 5′-fluorescence (TET)-labeled damaged DNA (ITR-60) was incubated with GST·XPC-hHR23B (Fig. 8, *lanes 3–5*), XPA + RPA (Fig. 8, *lanes 6–8*), or all three proteins (Fig. 8, *lanes 9–11*), and 20 min later, the remaining factor(s) were added to the reaction mixtures. The mixtures were then analyzed by the GST·XPC pull-down assay for the interaction of GST·XPC-hHR23B with damaged DNA. Regardless of the order of assembly, all assembly groups showed remarkably similar patterns in that the addition of increasing amounts of XPA + RPA proportionally displaced XPC-hHR23B from damaged DNA (Fig. 8). This result not only supports the multistep damage recognition in NER, but is also in keeping

with a notion that XPC-hHR23B is the global initiator in the damage recognition process.

DISCUSSION

Recognition of damaged DNA is a complex process involving a number of proteins (XPA, RPA, XPC-hHR23B, and TFIIH), all of which can independently bind to the damaged DNA (Refs. 1–4 and references therein). Although some biochemical properties of damage recognition proteins are known, the molecular mechanism of how these proteins function at the damaged DNA site is not clear. In this study we carried out a comprehensive biochemical analysis on the interaction of damage recognition factors themselves and with damaged DNA.

XPC as a Global Initiator in NER—Recent *in vitro* studies strongly point to a role for XPC-hHR23B as the initiator of global genomic repair (25). This is primarily based on the findings that: 1) preincubation of UV-damaged plasmid DNA with XPC was preferentially repaired in an *in vitro* kinetic experiment, and 2) XPC shows a considerable preference for binding to UV-damaged DNA in the presence of nondamaged competitor DNA (24, 25). On the other hand, a separate *in vitro* study demonstrated that preincubation of damaged DNA with RPA and XPA, compared with that of XPC-hHR23B, led to a faster repair (10).

From the study described here and the previous studies by others (9, 16, 19–23), it is evident that XPC-hHR23B and RPA share basic properties in damage recognition: 1) preferential binding to the damaged DNA, and 2) the physical interaction with XPA (11–13) (Fig. 3), which makes both XPC-hHR23B and RPA eligible for a global initiator in NER (10, 19). On the other hand, these two proteins exhibit quite different biochemical characteristics: first, XPC has a considerable preference for binding to UV- or cisplatin-damaged DNA in the presence of nondamaged competitor DNA (24, 25) (Fig. 2*B*), whereas RPA retains only a moderate preference to damaged DNA over nondamaged DNA (16). Second, RPA has a significant preference to ssDNA over dsDNA, whereas XPC (or XPC-hHR23B) shows a higher affinity to dsDNA over ssDNA (35) (Fig. 2). The latter finding suggests that XPC-hHR23B functions at an early stage of duplex DNA damage recognition, whereas RPA is involved in a later stage of damage recognition including a structural distortion of damaged DNA that leads to an unwinding of duplex DNA (or a generation of ssDNA). Third, XPC-hHR23B and RPA differ in their interaction with XPA such that the RPA-XPA interaction stabilizes weak binding of XPA to the damaged DNA (14, 16) (Fig. 4) necessary for NER action (14), whereas the interaction between XPC-hHR23B and XPA did not contribute to a stability of the XPA-damaged DNA complex (Fig. 4). Instead, the presence of XPC-hHR23B interferes with the formation of the RPA-XPA-damaged DNA complex (Fig. 4). Moreover, the XPA-XPC interaction may eventually lead to the displacement of XPC-hHR23B from damaged DNA (Fig. 5). The difference between XPC-hHR23B and RPA in their biochemical characteristics described above not only reflects their unique role(s) in the early stage of NER, but also supports the role for XPC-hHR23B as a global initiator in NER.

Multistep Damage Recognition Process in Early Stage of NER—XPC physically interacted with XPA (Fig. 3), but the interaction was significantly inhibited by the presence of damaged DNA (Fig. 4). This observation suggests that the interaction between XPA and XPC may be necessary to recruit XPA to the damaged DNA site. The XPC-hHR23B, once binding to the damaged DNA, likely recruits XPA (Fig. 3) and other repair factors such as TFIIH and XPG (38) to the damaged DNA site (Fig. 9). XPC-hHR23B is also involved in the recruitment of TFIIH to damaged DNA (26). The XPA-RPA, once introduced to the damaged DNA site, cooperates with each other to destabilize the XPC-hHR23B-damaged DNA interaction. A recent *in vitro* study also supported the absence of XPC-hHR23B in the final incision complex (39). Displacement of XPC-hHR23B from the damaged DNA likely requires a physical interaction between XPA and RPA on damaged DNA because a mutant RPA lacking XPA interaction domain poorly functioned in the displacement of XPC from damaged DNA (Fig. 7). The XPA-RPA interaction on the damaged DNA would likely force XPC to dissociate itself from XPA and the damaged DNA (Figs. 5 and 9). These lines of evidence suggest that XPA, RPA, and XPC-hHR23B do not form a stable three-protein complex at the damaged DNA site. Instead, it supports a notion that the damage recognition process occurs in a stepwise manner. However, we cannot rule out a possibility that XPA, XPC-hHR23B, and RPA form a transient complex on the damaged DNA prior to the displacement of XPC-hHR23B (Fig. 9) because RPA and XPC likely recognize two separate domains of XPA (Fig. 3). We should also point out that the *in vitro* study described here did not include TFIIH, a key damage recognition protein involved in distinguishing the damaged strand from the nondamaged one (27) as well as local unwinding of the damaged DNA region. The interaction between XPC and TFIIH appears to be essential for nucleotide excision repair (40). Nonetheless, addition of TFIIH appeared to have no effect on the RPA/XPA-mediated

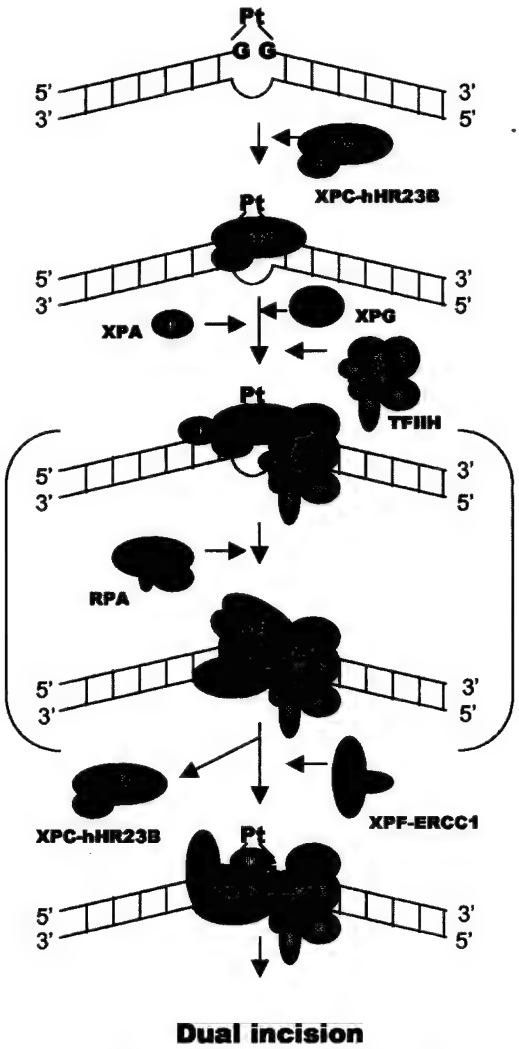


FIG. 9. **A proposed molecular mechanism of damage recognition process in the early stage of nucleotide excision repair.** Transient steps are indicated with *brackets*.

displacement of XPC-hHR23B from damaged DNA.[2]

Role for hHR23B in Damage Recognition—The XPC forms a stable complex with hHR23B (41, 42). Although the XPC subunit is solely responsible for the binding of the XPC-hHR23B complex to the damaged DNA (23) and the interaction with XPA (Fig. 3), hHR23B (Rad23) is essential for XPC function in NER (23). Very large amounts of XPC without hHR23B showed some repair activity, but equimolar hHR23B led about 10-fold higher activity (20). Rad23 contains a ubiquitin-associated domain that may play a role in controlling NER through proteosome-mediated degradation of repair factors (33, 43). In addition, hHR23B interacts with the base excision repair protein, *N*-methylpurine-DNA glycosylase, suggesting that it may have a role in mediating various repair pathways (37). In this study we found that hHR23B is necessary for XPA/RPA-mediated displacement of the XPC-hHR23B complex from damaged DNA (Fig. 6). hHR23B does not directly interact with XPA or RPA; we do not know what specific role hHR23B plays in the displacement of XPC from damaged DNA. A recent study showed that the DNA binding domain of XPC overlaps with the hHR23B interaction domain (40), suggesting that hHR23B may facilitate the displacement of XPC through the modulation of its DNA binding activity in the presence of XPA + RPA (Fig. 3).

Physiologic significance of the displacement of XPC from damaged DNA in NER is yet to be determined, however, it at least provides a crucial information that damage recognition occurs in an ordered, multistep process. Our finding that the order of protein assembly had no effect on the displacement of XPC from damaged DNA (Fig. 8) supported a notion that XPC-hHR23B is the initiator of global genomic repair (19, 24). Because XPC, compared with RPA and XPA, exhibited exceptionally strong affinity to damaged DNA (24), it is quite possible that the role for XPC-hHR23B is to effectively identify DNA damage *in vivo*. RPA-XPA-mediated displacement of XPC may be necessary for the formation of the stable XPA·RPA complex at the damaged site, which would allow a proper positioning of the endonucleases (XPG and ERCC1-XPF) for accurate and efficient incisions. It should be pointed out, however, that the RPA-XPA-mediated displacement of XPC from damaged DNA could be a part of the alternative global genomic NER. For example, although XPC-HR23B does not preferentially bind to CPD DNA (24), repair of *cis-syn* cyclobutane dimer-containing DNA was dependent on XPC-hHR23B, suggesting that there may be alternative pathways for the global genomic NER that requires XPC-HR23B but can be replaced by XPA and RPA (30). Further functional analysis would be necessary to validate the role for damage recognition complex in NER action.

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[2] J-S. You and S-H. Lee, unpublished data.

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Role of Zinc-Finger Motif in Redox Regulation of Human Replication Protein A

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ABSTRACT

Replication protein A (RPA) is a heterotrimeric zinc-finger protein complex involved in DNA replication, repair, and genetic recombination. Unlike other zinc-finger proteins, RPA's zinc-finger motif is not essential for its single-stranded DNA (ssDNA) binding activity, but is involved in redox regulation of its single-stranded DNA (ssDNA) binding activity. To get an insight into the regulation of RPA–ssDNA interaction, wild-type RPA (wt-RPA) and zinc-finger mutant were examined for ssDNA binding activity using surface plasmon resonance technique. Interaction of wt-RPA with ssDNA under nonreducing conditions was very weak ($K_D \times 2.33 \times 10^{-8}$ *M*) compared with that under reducing conditions ($K_D = 7.35 \times 10^{-11}$ *M*), whereas ssDNA binding affinity of the zinc-finger mutant was not affected by redox. The divalent ion chelator, *o*-phenanthroline, significantly reduced wt-RPA–ssDNA interaction, but had no effect on the zinc-finger mutant. The inhibitory effect of *o*-phenanthroline on RPA–ssDNA interaction was reversed by Zn(II), but not by other divalent cations, suggesting that Zn(II) is the unique metal coordinating the zinc-finger cysteines in redox regulation of RPA–ssDNA interaction. In DNA repair, redox affected RPA's interaction with damaged DNA, but not its role in stabilizing the xeroderma pigmentosum group A (XPA)-damaged DNA complex, suggesting that the zinc-finger motif may mediate the transition of RPA–XPA interaction to a stable RPA–XPA-damaged DNA complex in a redox-dependent manner. Antioxid. Redox Signal. 3, 657–669.

INTRODUCTION

REPLICATION PROTEIN A [RPA; also known as human single-stranded DNA (ssDNA) binding protein, HSSB] is a three-subunit protein complex consisting of 70-, 34-, and 11-kDa subunits involved in DNA replication, repair, and genetic recombination (Fig. 1A) (28). In DNA replication, RPA is involved in unwinding of the replication origin, whereas it interacts with the DNA polymerase α-primase complex at the origin (5, 7, 15, 20). In nucleotide excision repair (NER), RPA interacts with the xeroderma pigmentosum group A complementing protein (XPA) on damaged DNA and stimulates XPA–DNA interaction and also recruits other repair proteins such as XPG, excision repair cross complementing protein 1 (ERCC1)-XPF, and transcription factor IIH (TFIIH) to the damaged site for the subsequent incision/excision step (11, 19). RPA may also be involved in a later stage of NER, gap-filling, that requires PCNA, RF-C, and DNA polymerase δ (or ε) (1).

The large subunit of RPA (p70) contains the ssDNA-binding domain that resides in the middle region of p70 with two homologous subdomains in tandem positions (3, 8, 14, 16). RPA p70 has a 4-cysteine type zinc-finger motif toward the C-terminus (amino acids

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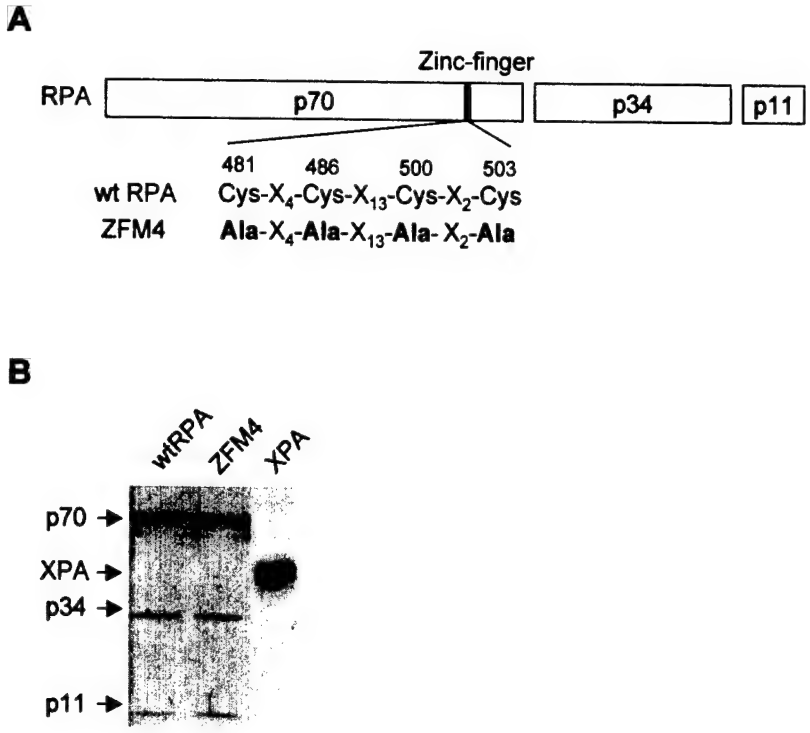


FIG. 1. Proteins used for this study. (**A**) Schematic representation of wild-type RPA (wt-RPA) and a zinc-finger mutant RPA [ZFM4; containing cysteine-to-alanine substitution at all four cysteine sites (amino acids 481, 486, 500, and 503)]. (**B**) SDS-PAGE of purified proteins. Proteins were separated by 12% SDS-PAGE followed by Coomassie Blue staining (see Materials and Methods).

481–503; Fig. 1A) (21, 30). Unlike other zinc-finger proteins, RPA's zinc-finger domain is not essential for its ssDNA binding activity and has little or no effect on its DNA binding activity (14, 16). Recent studies have shown that RPA's ssDNA binding activity is regulated by redox through the cysteines in a putative zinc-finger domain (21, 30). In fact, all four zinc-finger cysteines are required for redox regulation of RPA's DNA binding activity (30). Under reduced condition, the zinc-finger structure is favorably formed and Zn(II) may protect the zinc-finger cysteines from engaging in disulfide bond formation. Under nonreducing conditions, however, oxidation of the Zn(II)-thiolate bond induces the release of Zn(II) from the pocket (21), which promotes the formation of disulfide bonds (21, 30). The redox status of RPA significantly affected initial interaction with ssDNA, but had no effect after RPA formed a stable complex with DNA (30), suggesting that redox regulation of the zinc finger may be involved in mediating initial RPA–ssDNA interaction to form a stable RPA–ssDNA complex.

XPA is also a zinc-finger protein involved in the damage recognition step of NER (10, 13, 24). XPA preferentially binds to (6-4) photoproduct of ultraviolet ray (UV)-damaged DNA and may also play a role in subsequent steps of NER through interaction with other repair proteins (13). XPA exhibits a low-affinity binding to UV-damaged DNA; hence, the interaction between XPA and RPA stimulates the XPA-damaged DNA complex and also recruits other repair proteins such as XPG, ERCC1-XPF, and TFIIH to the damaged site for the subsequent incision/excision step (11, 19, 27). A mutation of RPA at any zinc-finger cysteine abolished its function in repair (6), suggesting a unique role for the zinc-finger domain in the early stage of repair.

To understand the role of the zinc-finger motif in the redox regulation of RPA–DNA interaction, we carried out a real-time analysis of

the interaction between RPA (and XPA) and DNA using the surface plasmon resonance (SPR) technique. We found that interaction of wild-type RPA (wt-RPA) with ssDNA under nonreducing conditions was 1/3,000th of the affinity observed under reducing conditions, whereas the zinc-finger mutant exhibited a strong binding to ssDNA regardless of its redox status. RPA-damaged DNA interaction was also regulated by redox, although RPA's role in stabilization of the XPA-damaged DNA complex was not affected by redox, suggesting that the zinc-finger motif may mediate the transition of RPA–XPA interaction to a stable RPA–XPA-damaged DNA complex in a redox-dependent manner.

MATERIALS AND METHODS

Proteins and antibodies

wt-RPA and a zinc-finger mutant [ZFM4; the mutation with the change of all four cysteines (the sites 481, 486, 500, and 503) into alanines] were prepared according to the procedure described previously with slight modifications (6). In brief, cell lysates were prepared from insect cells (Sf-9), coinfected with recombinant baculoviruses encoding three subunits (11 kDa, 34 kDa, and either wild-type or zinc-finger mutant 70 kDa). After the salt concentration (0.5 *M* NaCl) was adjusted, cell lysates were loaded onto a ssDNA cellulose column (1.0 × 8 cm) equilibrated with buffer A [25 m*M* Tris-HCl (pH 7.5), 10% glycerol, 0.02% Nonidet P-40, 1 m*M* dithiothreitol (DTT), 0.5 m*M* EDTA, 0.1 m*M* phenylmethylsulfonyl fluoride, 0.1 μg/ml leupeptin, and 0.2 μg/ml antipain] containing 0.5 *M* NaCl. The column was successively washed with 20 column volumes of buffer A containing 0.5 *M* NaCl and 0.8 *M* NaCl. The proteins were eluted with buffer A containing 2.0 *M* NaCl and 40% ethylene glycol. The eluted fractions were diluted fivefold with buffer A, and loaded onto an Affi-Gel Blue (Bio-Rad) column equilibrated with buffer A containing 0.5 *M* NaCl. After the column was washed with buffer A containing 0.5 *M* NaCl and 0.8 *M* NaCl, proteins were eluted with buffer containing 2.5 *M* NaCl and 40% ethylene glycol. The RPA-containing fractions were pooled and dialyzed against buffer A containing 50 m*M* NaCl and further purified on a Q-Sepharose column with a linear salt gradient (50 m*M* to 0.4 *M* NaCl). During purification, RPA was monitored by immunoblotting using anti-p70 and -p34 antibodies (25). Histidine-tagged XPA was prepared according to the procedure described previously (15).

Biomolecular interaction analysis

Interaction of wt-RPA , a zinc-finger mutant, and/or XPA with ssDNA (or UV-damaged DNA) was monitored using an SPR biosensor instrument, Biacore 3000 (Biacore) as described previously (27). For preparation of the biosensor surface with DNA, 5′-biotinylated 70mer DNA was diluted to 1.5 n*M* in a buffer containing 10 m*M* sodium acetate (pH 4.8) and 1.0 *M* NaCl, and manually injected onto an immobilized streptavidin surface of the Biacore sensor chip to the desired density in different flow cells. One flow cell was left underivatized to allow for refractive index change correction. Proteins were diluted in the running buffer containing 10 m*M* HEPES (pH 7.4), 150 m*M* NaCl, 2 m*M* $MgCl_2$, 0.005% polysorbate-20, and 1 m*M* DTT. Each experiment was repeated at least twice to assure reproducibility.

Electrophoretic mobility shift assay of RPA–ssDNA interaction

Oligo$(dT)_{50}$ was labeled with [γ-^{32}P]ATP (ICN) and T4 polynucleotide kinase (Roche Molecular Biochemical) based on the manufacturer's instructions. The indicated amount of wt-RPA or zinc-finger mutant was incubated with 100 fmol of 5′-^{32}P-labeled oligo$(dT)_{50}$ at room temperature for 25 min in a reaction mixture containing 50 m*M* HEPES-KOH (pH 7.8), poly(dI-dC) (0.2 μg), bovine serum albumin (0.2 μg/μl), 200 m*M* NaCl, and the indicated amounts of other chemicals. The RPA–ssDNA complex was analyzed on 5% polyacrylamide gels in 0.5X Tris–borate–EDTA buffer (acrylamide: bisacrylamide = 43.2:0.8). The gels were dried and exposed to x-ray films (Kodak). For quantification, the bands of interest were excised

from the gels and measured for radioactivity using a Beckman Scintillation Counter LS 6500.

Proteolytic analysis of wt-RPA and zinc-finger mutant with trypsin

The reaction mixture (60 μl) contained 0.1 *M* Tris-HCl (pH 8.5), 120 ng/μl wild-type or mutant RPA, and, where indicated, 200 ng/μl oligo(dT)$_{50}$. After the reaction mixture was incubated at room temperature for 15 min, 50 ng of trypsin (sequencing grade; Boehringer Mannheim) was added into the reaction. The reaction mixture was then immediately incubated at 37°C. Eight or 10 μl of the sample was removed from the reaction at the indicated time points. The reaction samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and were analyzed by the western blot (Fig. 1B).

Western blotting

Western blots were performed as described previously (25). In brief, wild-type or mutant RPA was run in a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane (BA83; Bio-Rad). After the transfer, either anti-p70 or anti-p34 polyclonal antibody was used to detect RPA subunits, which were then revealed by ^{125}I-protein A (Amersham Corp.) treatment and visualized by autoradiography.

Circular dichroism (CD) analysis

CD spectra of purified wt-RPA and a zinc-finger mutant were acquired on a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co.). Far-UV CD spectra (200–260 nm) were measured in a 1-mm path length quartz cell and represent averages of four accumulations with a protein concentration of 100 μg/ml in 25 m*M* Tris-HCl (pH 7.5), 10% (vol/vol) glycerol, 0.02% (vol/vol) NP40, 0.5 m*M* EDTA, 0.1 m*M* phenylmethylsulfonyl fluoride, 0.1 μg/m*M* leupeptin, 0.2 μg/ml antipain, and 200 m*M* NaCl. All spectra were baseline-corrected by subtraction of an averaged scan derived from the buffer alone to obtain the mean molar residue ellipticity (deg cm^2 dmol^{-1}). The result from CD spectra was deconvoluted to obtain information on the secondary structures that are mostly affected by the zinc-finger motif during interaction of protein with damaged DNA. The software program (CDnn) used to deconvolute CD spectra was obtained through the Internet (http://bioinformatik.biochemtech.uni-halle.de/cdnn/).

RESULTS

Role of zinc-finger motif in redox regulation: kinetic analysis of RPA's ssDNA binding activity

Our recent study indicates that all four zinc-finger cysteines are essential for redox regulation of RPA's–ssDNA binding activity (30). Redox regulation affects initial RPA-ssDNA interaction, but has no effect after RPA forms a stable complex with DNA (30), suggesting that the zinc-finger motif may mediate the initial interaction of RPA with ssDNA. To get an insight into the RPA–ssDNA interaction, we used the SPR technique to examine the ssDNA binding activity of wt-RPA and zinc-finger mutant (ZFM4) under various redox conditions, which allows macromolecular interactions to be measured in real time (27). wt-RPA or zinc-finger mutant ranging from 0.125 n*M* to 2 n*M* was injected onto a biosensor chip containing a low level (33 RU) of 70mer ssDNA for 600 s, followed by 900 s of a buffer injection period for dissociation. The sensorgram indicated that both wt-RPA and zinc-finger mutant bound to ssDNA with high affinity in the presence of 1 m*M* DTT (Fig. 2B and D). Under nonreducing conditions, however, wt-RPA exhibited very low ssDNA binding activity, whereas ZFM4 maintained its high-affinity binding to ssDNA (Fig. 2A and C, and Table 1). Interaction of wt-RPA with the ssDNA surface under nonreducing conditions was very weak, as indicated by the derived $K_D = 2.33 \times 10^{-8}$ *M*, 1/3,000th of the affinity observed for RPA under reducing conditions (Table 1). Even though wt-RPA exhibited a low-affinity binding to ssDNA under nonreducing conditions, it maintained a stable interaction with ssDNA once forming a complex.

A unique role for Zn(II) in RPA–ssDNA interaction

To understand the role of Zn(II) in the redox regulation of RPA–ssDNA interaction, we ex-

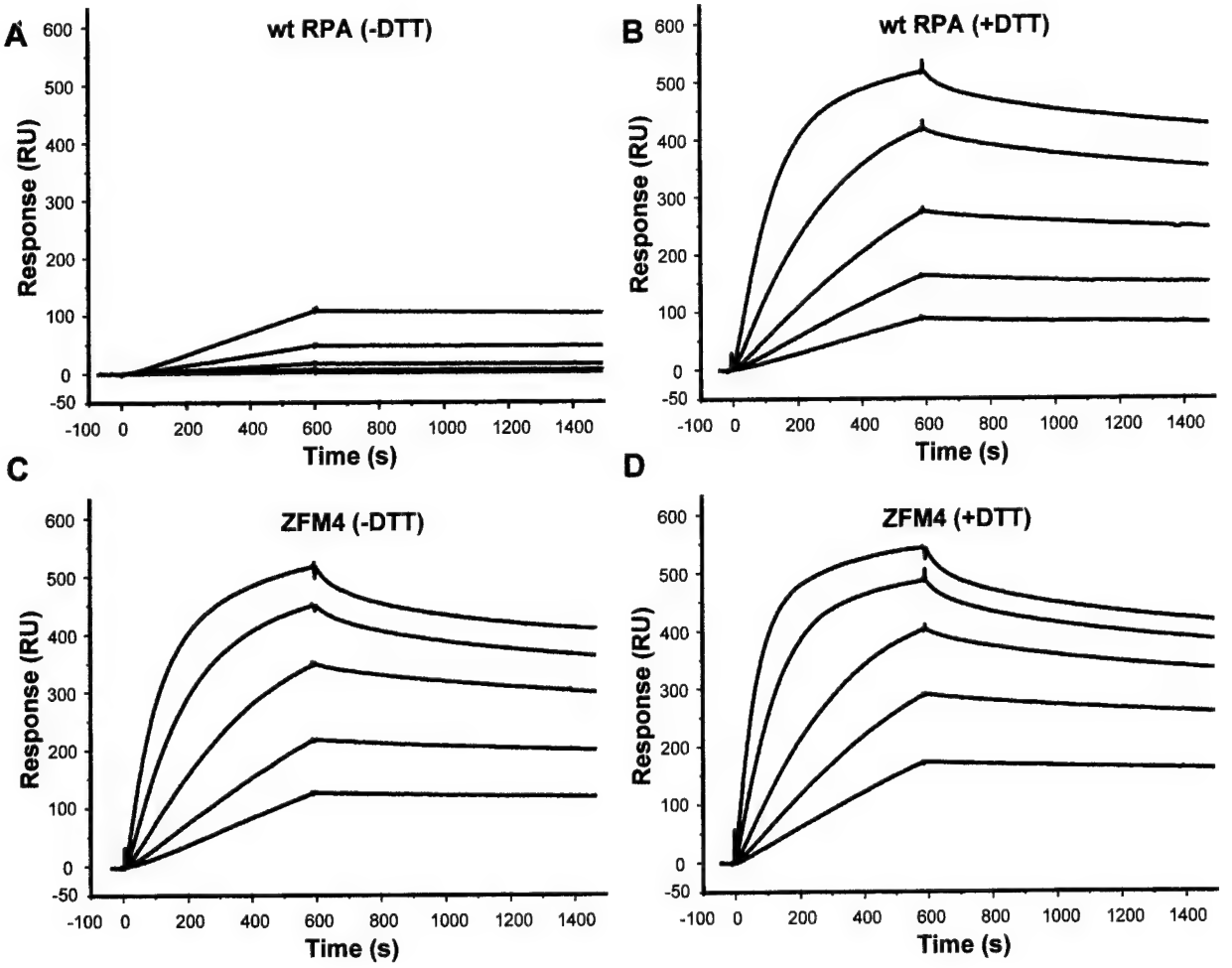


FIG. 2. Biomolecular interaction analysis of RPA–ssDNA binding under redox conditions. A 5 n*M* concentration of wt-RPA or a zinc-finger mutant (ZFM4) was injected onto a sensor chip surface containing 33 RU of ssDNA using the KINJECT function of Biacore 3000. The association phase was allowed for 600 s followed by 900 s of a buffer injection period for dissociation. (**A**) wt-RPA in the absence of DTT. (**B**) wt-RPA in the presence of 1 m*M* DTT. (**C**) ZFM4 in the absence of DTT. (**D**) ZFM4 in the presence of 1 m*M* DTT.

amined various divalent ions for RPA's ssDNA binding activity (Fig. 3). The role of Zn(II) in RPA-ssDNA interaction was supported by the fact that the inhibitory effect of the divalent ion chelator, *o*-phenanthroline, was reversed by addition of Zn(II) (30; Fig. 3A, lanes 4–6). In contrast, the interaction of the zinc-finger mutant with ssDNA was not affected by either *o*-phenanthroline or Zn(II) (Fig. 3A, lanes 7–16). Addition of an excess amount of Zn(II) nonspecifically inhibited RPA–ssDNA interaction regardless of *o*-phenanthroline's presence, suggesting that Zn(II)-dependent reversal of the inhibitory effect of *o*-phenanthroline functions within stoichiometric concentration (data not shown). To assess the role of Zn(II) in coordination of the zinc finger cysteine, we examined other divalent ions to see whether they can replace Zn(II) in RPA–ssDNA interaction (Fig. 3B). Mg(II), Ca(II), and Cu(II) were not able to compensate for Zn(II) in overcoming the inhibitory effect of *o*-phenanthroline in RPA–ssDNA interaction, whereas Fe(II) partially replaced Zn(II) (Fig. 3B). This result suggests that Zn(II) is the unique metal element coordinating four cysteine residues at RPA's zinc-finger motif.

Involvement of zinc-finger domain in RPA's structural change following interaction with DNA

To understand further the role of the zinc-finger motif in RPA–ssDNA interaction, we ex-

TABLE 1. EQUILIBRIUM AND KINETIC BINDING CONSTANTS OF WT-RPA AND A ZINC-FINGER MUTANT (ZFM4) TO SSDNA

RPA	K_D(M)	$k_a(s^{-1}\ M^{-1})$	$k_d(s^{-1})$
+ DTT			
Wild-type	$(7.35 \pm 0.01) \times 10^{-11}$	$(2.76 \pm 0.02) \times 10^{-6}$	$(2.03 \pm 0.01) \times 10^{-4}$
ZFM4	$(4.47 \pm 0.04) \times 10^{-11}$	$(6.39 \pm 0.01) \times 10^{-6}$	$(2.86 \pm 0.02) \times 10^{-3}$
− DTT			
Wild-type	$(2.33 \pm 0.20) \times 10^{-8}$	$(2.38 \pm 0.06) \times 10^{-3}$	$(5.53 \pm 0.32) \times 10^{-5}$
ZFM4	$(4.97 \pm 0.03) \times 10^{-11}$	$(4.49 \pm 0.01) \times 10^{-6}$	$(2.23 \pm 0.01) \times 10^{-4}$

The data were globally fit to a 1:1 binding model.

amined far-UV CD spectra of wt-RPA and a zinc-finger mutant to see a change in secondary structure upon binding to DNA. A significant change in CD spectra was observed with both wt-RPA and a zinc-finger mutant upon binding to DNA (Fig. 4), suggesting that RPA undergoes a significant conformational change upon binding to DNA. A noticeable difference between wt-RPA and a zinc-finger mutant in the CD spectra pattern was observed under reducing conditions (Fig. 4A and C, and Table 2). Under nonreducing conditions, however, very little change in CD spectra between wt-RPA and zinc-finger mutant was observed (Fig. 4B and C, and Table 2). This result not only supports a role for the zinc-finger domain in the structural change of RPA, but also establishes a relationship between redox regulation and the structural change of RPA upon binding to DNA.

We also examined p70 and p34 subunits of wt-RPA and zinc-finger mutant for the tryptic digestion patterns in the presence and absence of ssDNA. In the absence of ssDNA, wt-RPA was cleaved into several tryptic products (55-, 47-, 19–21-, and 25-kDa fragments), whereas 19 kDa and 55 kDa were the major cleavage fragments in the presence of ssDNA (Fig. 5A). This result is in keeping with that from far-UV CD spectra analysis (Fig. 4) and suggests that RPA undergoes a conformational change upon binding to DNA (8, 21). The zinc-finger mutant, however showed very little change of its tryptic digestion in the presence of ssDNA (Fig. 5B), suggesting that a mutation at zinc-finger cysteine affects p70's conformational change upon binding to ssDNA. We also examined the tryptic digestion pattern of p34 to see whether the mutation at p70's zinc-finger domain affects the conformation of the p34 subunit. The p34 subunit of the zinc-finger mutant (ZFM4) was not only protected from tryptic digestion, but also unaffected by the presence of ssDNA (Fig. 5D). On the other hand, wt-RPA showed a change in the p34 tryptic digestion pattern upon binding to ssDNA, such that the p34 subunit was more accessible to tryptic digestion following RPA–ssDNA interaction. This result suggests that the mutation at the zinc-finger domain of p70 may also affect the accessibility of p34 to tryptic digestion.

Redox regulation of RPA's zinc-finger motif in recognition of damaged DNA

RPA not only has a preferential binding to UV- or cisplatin-damaged DNA, but also can stabilize XPA-damaged DNA complex through protein–protein interaction (2, 4, 22, 26, 27). Although zinc-finger mutant RPA failed to support NER activity (6), it also can stabilize the interaction of XPA with damaged DNA (26). We therefore examined whether redox affects RPA's interaction with damaged DNA and/or with XPA. Both XPA and wt-RPA are the 4-cysteine type zinc-finger proteins, and their interactions with UV-damaged DNA were affected by redox (Fig. 6A and B), whereas the interaction of the zinc-finger mutant RPA with damaged DNA was not affected by redox change (Fig. 6B). On the other hand, RPA's role in stabilization of XPA-damaged DNA complex was not affected by redox (Fig. 6A & 6B), suggesting that the failure of zinc-finger mutant in supporting NER may not be due to its role in interaction with damaged DNA or XPA. Redox regulation of the zinc finger significantly affected the initial RPA–ssDNA interaction, but

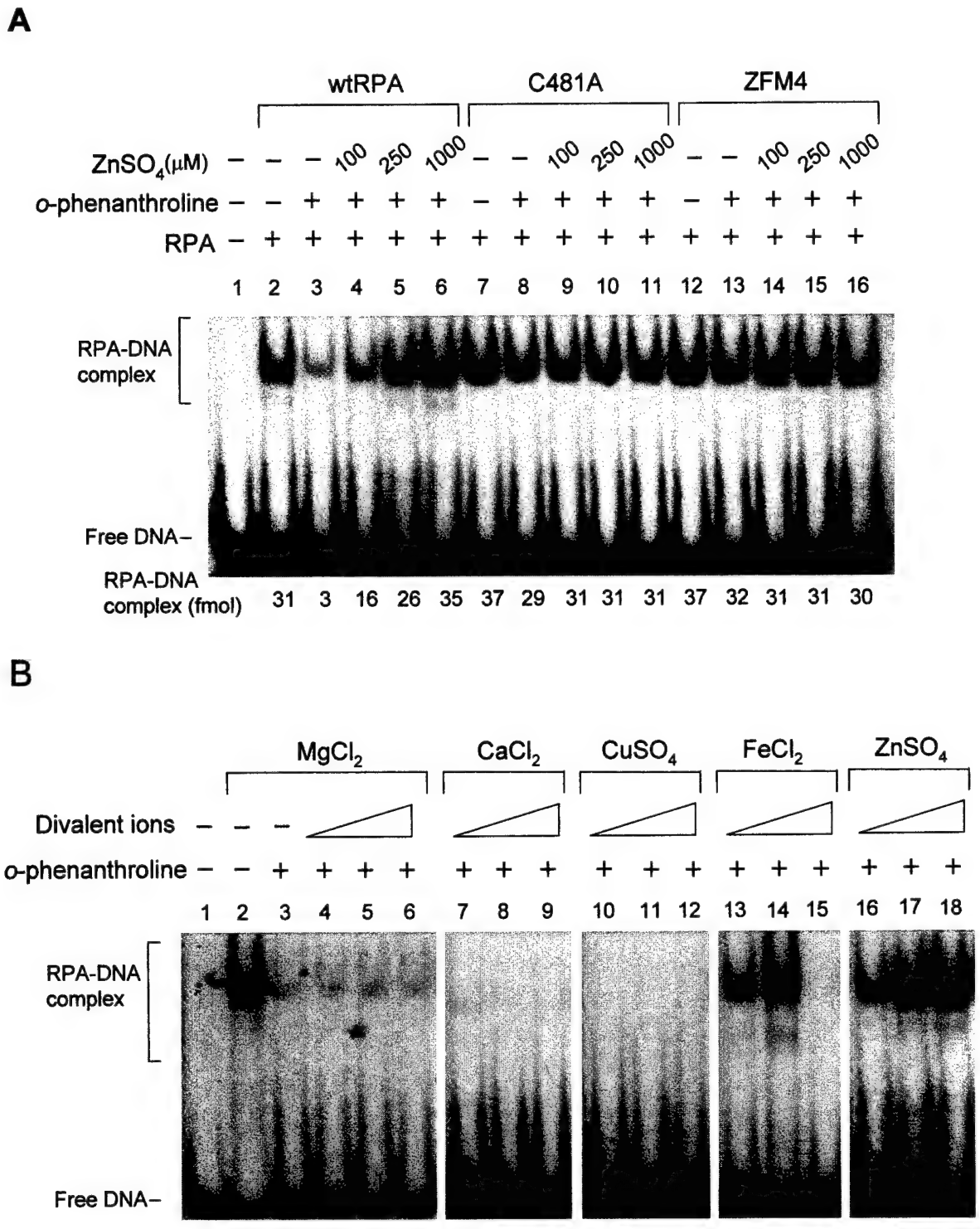


FIG. 3. (**A**) Inhibitory effect of *o*-phenanthroline on RPA ssDNA binding activity is restored by Zn(II). wt-RPA or zinc-finger mutant (20 ng) was treated with 1.0 m*M* *o*-phenanthroline followed by the addition of increasing amounts of Zn(II) to the reaction mixture. After incubation for 15 min in the presence of 100 fmol of 5′-^{32}P-labeled oligo(dT)$_{50}$, the RPA–ssDNA complex was analyzed by gel mobility shift assay (5% PAGE). Lane 1 contained no RPA. (**B**) Effect of various metal ions on RPA's ssDNA binding activity in the presence of *o*-phenanthroline. wt-RPA (20 ng) was treated with 1.0 m*M* *o*-phenanthroline and the indicated amounts of divalent metal (100 μM in lanes 4, 7, 10, 13, and 16; 250 μM in lanes 5, 8, 11, 14, and 17; 1,000 μM in lanes 6, 9, 12, 15, and 18) added to the reaction mixture before incubation for 15 min with 100 fmol of 5′-^{32}P-labeled oligo(dT)$_{50}$. No RPA was included in lane 1. The RPA–ssDNA complex was analyzed by the procedure as described in A.

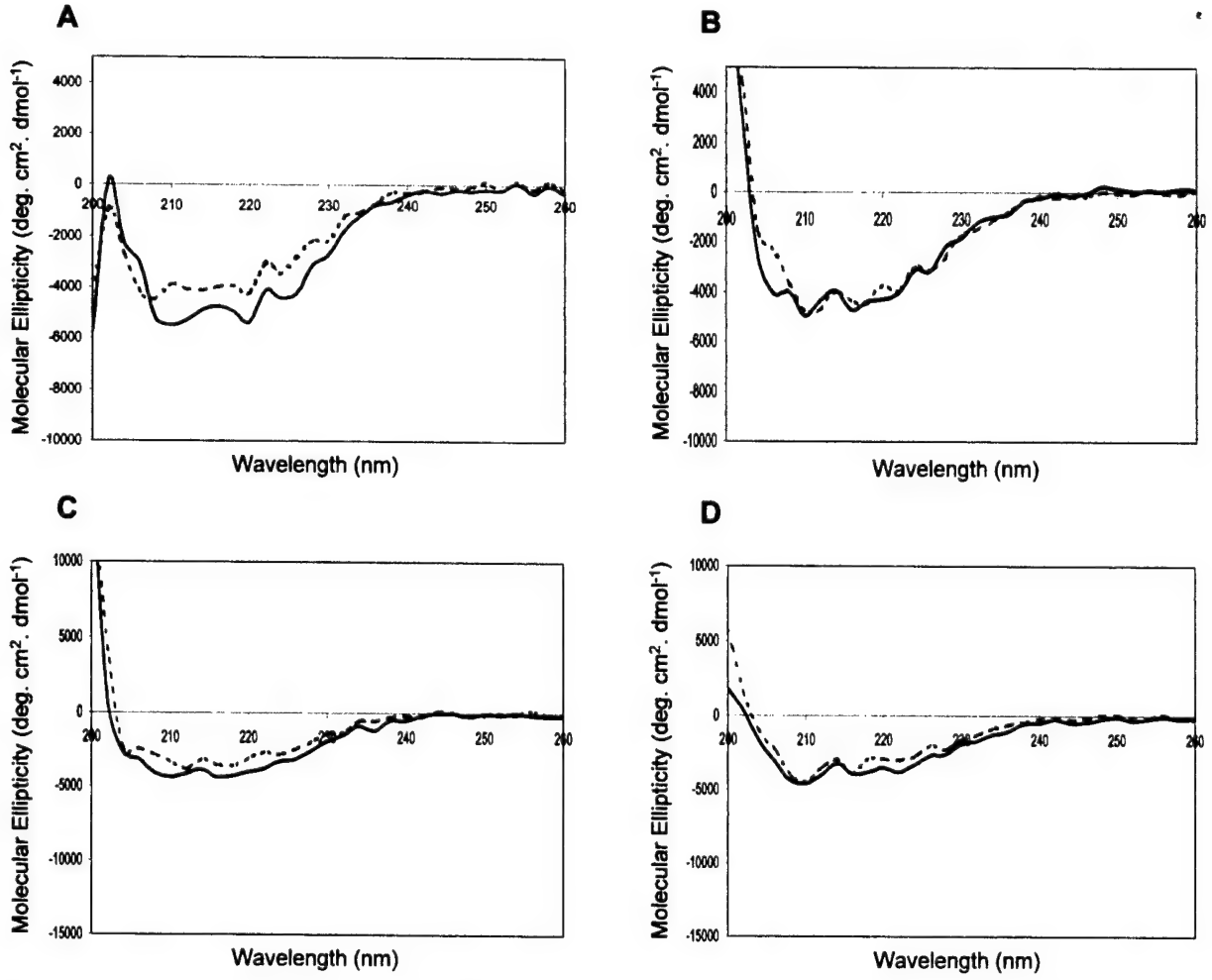


FIG. 4. Far-UV CD spectra of wild-type RPA (—) and zinc-finger mutant (ZFM4) (- - -) under various conditions. Reactions were carried out at 25°C in either the absence (**A** and **B**) or presence (**C** and **D**) of oligo(dT)$_{50}$. Reactions contained either 1 m*M* (**A** and **C**) or 0 m*M* DTT (**B** and **D**).

had no effect after RPA formed a stable complex with DNA (30). Similarly, RPA–XPA-damaged DNA complex, once formed, was not affected by redox (Fig. 6C), suggesting that the zinc-finger motif may mediate the transition of RPA–XPA interaction to a stable RPA–XPA-damaged DNA complex in a redox-dependent manner.

DISCUSSION

The zinc finger is one of the key structural motifs found in many DNA binding proteins in eukaryotes and implicates a role in the regulation of DNA binding activity through redox (9, 12, 29). Nonetheless, the role of the zinc finger in redox regulation has not been directly demonstrated because the zinc finger domain is essential for sequence-specific DNA binding and a mutation at this region would abolish DNA binding activity. RPA's zinc-finger motif is not a DNA binding element, and a mutation at the zinc-finger motif has very little effect on its DNA binding activity (8, 14, 16), which makes RPA an excellent model to study the role of zinc-finger motifs in redox regulation. In this study, we analyzed a role for RPA's zinc-finger motif in the redox regulation of RPA–DNA interaction.

The SPR analysis indicated that interaction of wt-RPA with ssDNA under nonreducing conditions was very weak and was only 1/3,000th of the affinity observed for RPA under reducing conditions (Table 1). Even though wt-RPA showed a low affinity to ssDNA un-

TABLE 2. FAR UV-CD ANALYSIS OF WT-RPA AND ZINC-FINGER MUTANT (ZFM)

	Helix (%)	*Antiparallel (%)*	*Parallel (%)*	*β-turn (%)*	*Random coil (%)*
wt-RPA (+ DTT)	10.9	29.6	5.5	20.3	36.1
wt-RPA (− DTT)	15.7	28.0	6.3	15.9	32.9
wt-RPA/DNA (+ DTT)	16.3	27.8	6.4	15.5	32.4
wt-RPA/DNA (− DTT)	11.4	31.1	5.8	18.5	35.0
ZFM (+ DTT)	9.9	31.4	5.4	20.3	36.3
ZFM (− DTT)	13.6	30.4	6.2	16.3	33.6
ZFM/DNA (+ DTT)	15.4	30.3	6.5	14.8	32.2
ZFM/DNA (− DTT)	11.7	32.3	6.0	17.4	34.4

der nonreducing conditions, it maintained a stable interaction with ssDNA once forming the RPA–ssDNA complex. This result is in keeping with a recent observation that redox regulation of the zinc finger significantly affected initial RPA–ssDNA interaction, but had no effect after RPA formed a stable complex with DNA (30). This result also suggests that

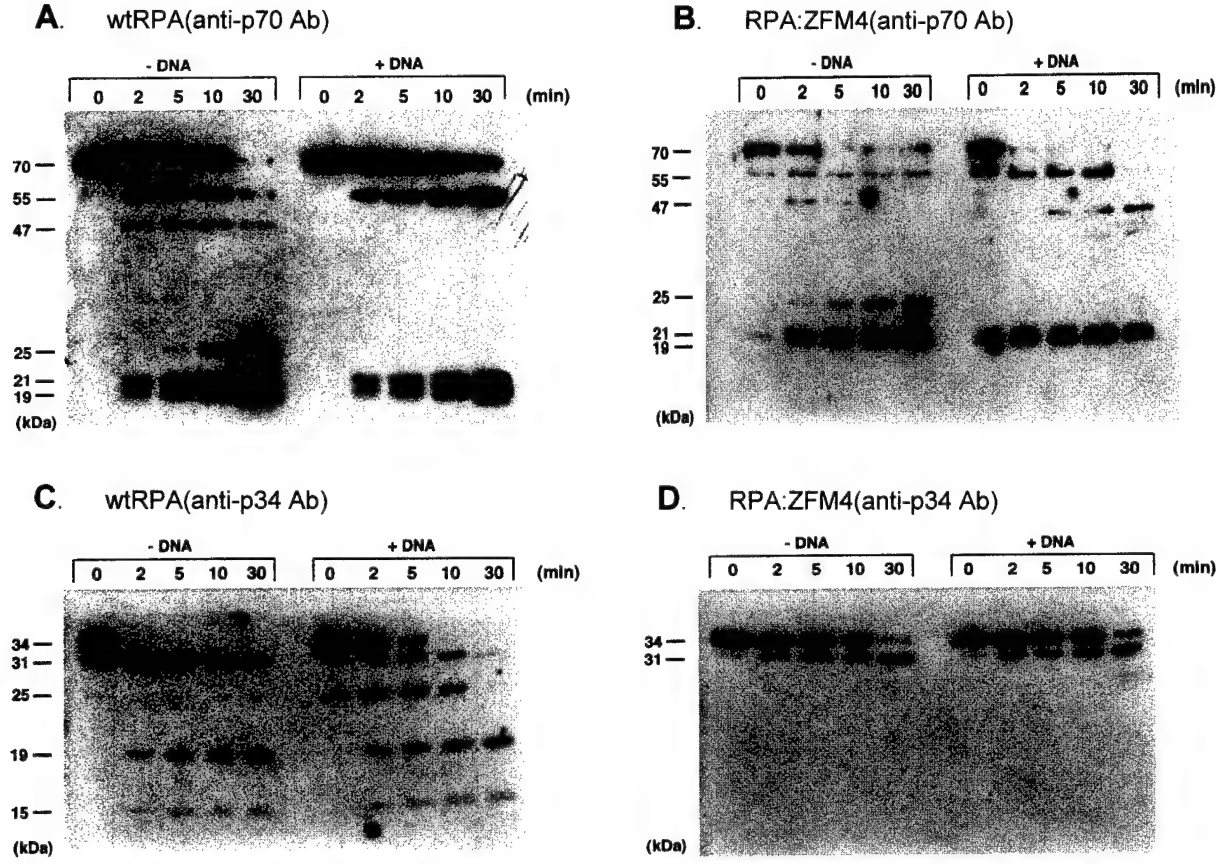


FIG. 5. Involvement of zinc-finger motif in the conformational change of RPA. (**A** and **B**) Tryptic digestion of RPA p70 subunit upon binding to ssDNA. Reaction mixtures (60 μl) contained 0.1 *M* Tris-HCl (pH 8.5) and 120 ng/μl of either wt-RPA (A) or a zinc-finger mutant (ZFM4) (B). Where indicated, 40 ng/μl oligo(dT) was added. After incubation at room temperature for 15 min, 50 ng of trypsin (sequencing grade; Boehringer Mannheim) was added to the reaction. Reaction mixtures were then incubated at 37°C, and aliquots were removed from the reactions at the indicated time points. Samples were mixed with gel loading buffer, boiled for 5 min, and loaded onto a 14% SDS-PAGE. The gel was then subjected to a western blot analysis using an anti-p70 polyclonal antibody. (**C** and **D**). Tryptic digestion pattern of RPA p34 subunit upon binding to ssDNA. Reaction mixtures and the sample preparations were the same as in A and B except that an anti-p34 polyclonal antibody was used to visualize p34 and its cleaved fragments for western analysis.

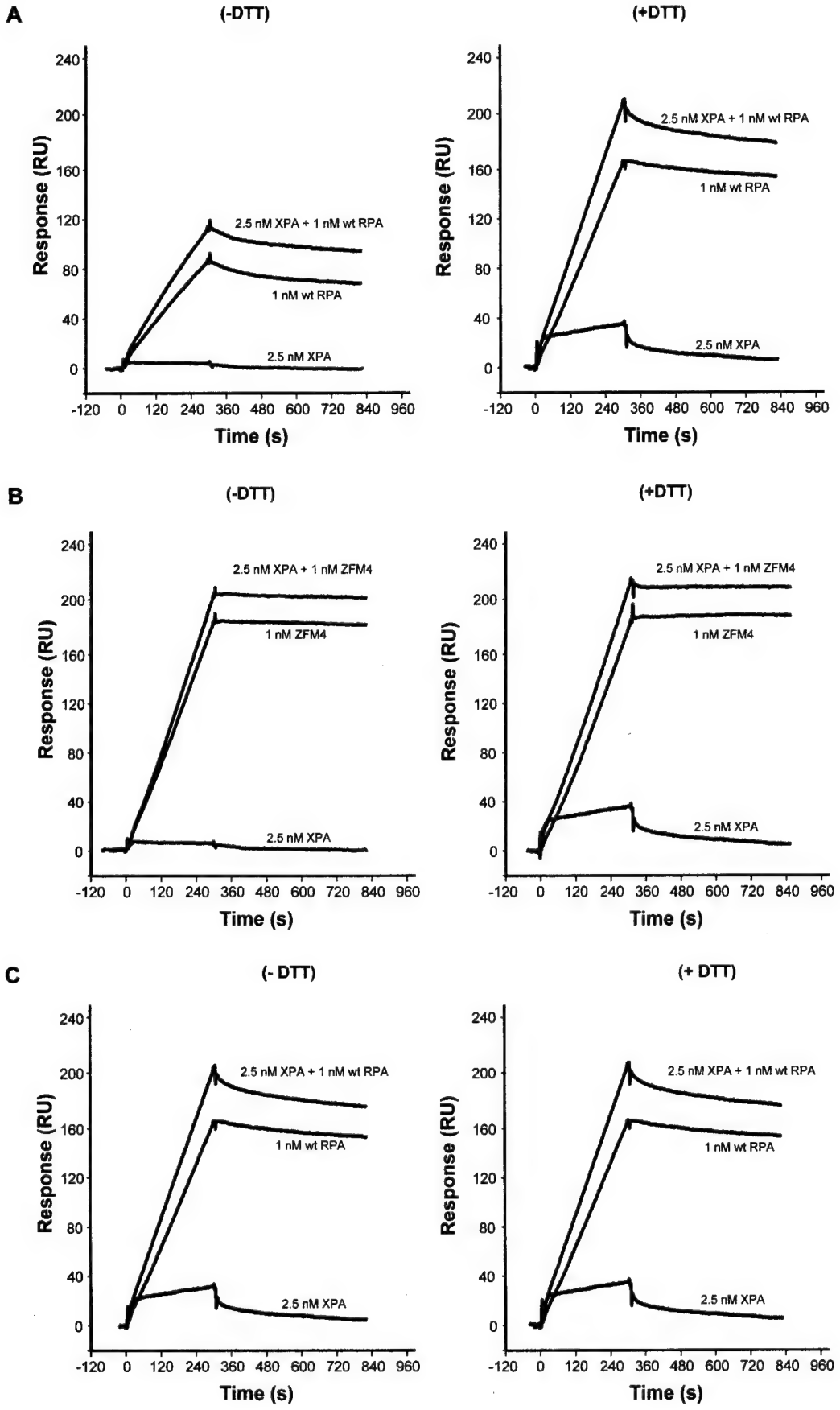
the zinc-finger motif mediates the transition of RPA–ssDNA interaction to a stable RPA–ssDNA complex in a redox-dependent manner.

The 4-cysteine zinc finger contains Zn(II) that tetrahedrally coordinates four cysteine residues (18). Under reducing conditions, the zinc-finger structure is favorably formed and Zn(II), buried in the interior, stabilizes the module by binding four cysteines (21), whereas the oxidation of the Zn(II)-thiolate bond induces the release of Zn(II) from the zinc finger, which likely promotes disulfide bond formation between the zinc-finger cysteines (21, 30). Other divalent ions were not able to compensate for Zn(II) in overcoming the inhibitory effect of *o*-phenanthroline in RPA–ssDNA interaction (Fig. 3), suggesting that Zn(II) may play a unique role in coordinating the zinc finger cysteines, which in turn controls RPA's ssDNA binding activity. The latter is also supported by the fact that a mutation at zinc-finger cysteines (amino acids 481, 486, 500, or 503) would be sufficient to abolish redox sensitivity of RPA's ssDNA binding activity (21, 30).

A significant change in the tryptic digestion pattern observed with wt-RPA, but not with a zinc-finger mutant, upon binding to ssDNA (Fig. 5A) strongly suggests that the conformational change of p70 occurs in an ssDNA-dependent manner, which mainly protects the large domain of p70 (55-kDa fragment) from proteolytic digestion. One possibility is that the 55-kDa fragment may actually be involved in the conformational change of p70 and protected from tryptic digestion through its interaction with ssDNA. It would be interesting to see whether the protected region of p70 (55-kDa fragment) represents the domain directly in contact with ssDNA. The p34 subunit (wt-RPA) also showed a change in the tryptic digestion pattern, becoming more accessible to tryptic digestion following its interaction with ssDNA, whereas the tryptic digestion pattern of the p34 subunit from the zinc-finger mutant (ZFM4) was virtually unaffected by the presence of ssDNA (Fig. 5D). This result suggests that the zinc-finger domain of p70 not only is essential for the conformational change of its own subunit, but may also affect the accessibility of p34 to tryptic digestion. The latter possibility was also supported by the previous finding where DNA-dependent protein kinase phosphorylated the p34 subunit from wt-RPA, but not that from the zinc-finger mutant, in the presence of ssDNA (6).

A role for RPA's zinc-finger domain has been demonstrated previously indicating that mutations at the zinc-finger domain differentially affected its function in DNA replication and NER (6, 17). The failure of zinc-finger mutants in supporting NER was not due to its role in the stabilization of the XPA-damaged DNA complex (26, 27). It is possible that the zinc-finger motif may be involved in mediating the generation of ssDNA during RPA-damaged DNA interaction (23). No matter what the role of RPA's zinc-finger domain in DNA repair may be, the initial interaction of XPA with damaged DNA under nonreducing conditions was significantly affected in the presence of wt-RPA, but not in the presence of a zinc-finger mutant (Fig. 6). It is not clear whether the differential effect of wt-RPA and a zinc-finger mutant on the XPA-damaged DNA interaction plays a direct role in NER. Considering the fact that the zinc-finger domain is involved in the conformational change of the RPA p70 subunit, it is conceivable that RPA's zinc-finger domain and its redox regulation may affect the early stage of repair, such as formation of a damage-recognition complex.

FIG. 6. Biacore analysis of redox regulation of RPA stabilization of XPA–(64) photoproduct interaction. wt-RPA or ZFM4 was injected onto a sensor chip surface containing 2,000 RU of double-stranded DNA, which contains a centrally located (6–4) photoproduct, using the KINJECT function of Biacore 3000. The association phase was allowed for 300 s followed by 540 s of a buffer injection period for dissociation. (**A**) wt-RPA (1 n*M*), XPA (2.5 n*M*), and the mixture of XPA (2.5 n*M*) and wt-RPA (1 n*M*) in the presence (1 m*M*) and absence of DTT. (**B**) ZFM4 RPA (1 n*M*), XPA (2.5 n*M*), and the mixture of XPA (2.5 n*M*) and ZFM4 RPA (1 n*M*) in the presence (1 m*M*) and absence of DTT. (**C**) wt-RPA (1 n*M*), XPA (2.5 n*M*), and the mixture of XPA (2.5 n*M*) and wt-RPA (1 n*M*) were injected onto the sensor chip surface in the presence of 1 m*M* DTT for a period of 300 s for association followed by a dissociation of 540 s by washing with a buffer containing either 1 m*M* or 0 m*M* DTT.


A
(-DTT)
(+DTT)
Response (RU)
Time (s)
2.5 nM XPA + 1 nM wt RPA
1 nM wt RPA
2.5 nM XPA
B
(-DTT)
(+DTT)
2.5 nM XPA + 1 nM ZFM4
1 nM ZFM4
2.5 nM XPA
C
(- DTT)
(+ DTT)
2.5 nM XPA + 1 nM wt RPA
1 nM wt RPA
2.5 nM XPA

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ABBREVIATIONS

CD, circular dichroism; DTT, dithiothreitol; ERCC1, excision repair cross complementing protein 1; NER, nucleotide excision repair; RPA, replication protein A; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; ssDNA, single-stranded DNA; TFIIH, transcription factor IIH; UV, ultraviolet ray; wt-RPA, wild-type RPA; XPA, xeroderma pigmentosum group A protein; ZFM, zinc-finger mutant RPA.

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Review

Recognition of DNA Damage in Mammals

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DNA damage by UV and environmental agents are the major cause of genomic instability that needs to be repaired, otherwise it give rise to cancer. Accordingly, mammalian cells operate several DNA repair pathways that are not only responsible for identifying various types of DNA damage but also involved in removing DNA damage. In mammals, nucleotide excision repair (NER) machinery is responsible for most, if not all, of the bulky adducts caused by UV and chemical agents. Although most of the proteins involved in NER pathway have been identified, only recently have we begun to gain some insight into the mechanism by which proteins recognize damaged DNA. Binding of *Xeroderma pigmentosum* group C protein (XPC)-hHR23B complex to damaged DNA is the initial damage recognition step in NER, which leads to the recruitment of XPA and RPA to form a damage recognition complex. Formation of damage recognition complex not only stabilizes low affinity binding of XPA to the damaged DNA, but also induces structural distortion, both of which are likely necessary for the recruitment of TFIIH and two structure-specific endonucleases for dual incision.

Keywords: DNA damage, Nucleotide excision repair, Replication protein A, XPA, and XPC-hHR23B

DNA damage recognition in nucleotide excision repair

Nucleotide excision repair (NER) action involves interaction of multiple proteins locating the damaged DNA sites, removal of short oligonucleotides containing DNA adducts, and synthesizing a replacement patch. In mammals, NER requires over 20 polypeptides including damage recognition/structure distortion factors [*Xeroderma pigmentosum* group A protein (XPA), XPC-hHR23B, replication protein A (RPA, also known as human single-stranded DNA binding protein, hSSB), and XPE], TFIIH containing two DNA helicases (XPB and XPD) that separate the strands to create an open preincision complex, two structure-specific endonucleases, ERCC1-XPF and XPG, and the enzymes necessary for filling-in the gap [DNA polymerase δ/ε, proliferating cell nuclear antigen (PCNA), replication factor C (RF-C), and RPA] (see Fig. 1).

An ongoing challenge is to understand how DNA damage is recognized and distinguished from non-damaged sites during NER. Recognition of DNA damage is the initial, but complicated event for NER. It involves multiple proteins: XPA, RPA, XPC-hHR23B, XPE and TFIIH, all of which can independently bind to damaged DNA and may have a role in damage recognition (Sancar, 1996; Dawn and Wood, 2000). Although some characteristics of the damage recognition proteins are known, the molecular mechanism of how these proteins function at the damaged DNA site is not clear. A recent *in vitro* study suggested that XPC-hHR23B is an initiator of global genomic repair, but not of transcription-coupled repair (Sugasawa *et al.*, 1998; Sugasawa *et al.*, 2001). This is based on the findings that preincubation of UV-damaged plasmid DNA with XPC was preferentially repaired in an *in vitro* kinetic experiment and also that XPC has considerable preference for binding to UV-damaged DNA in the presence of non-damaged competitor DNA (Sugasawa *et al.*, 1998). It should be noted however that the binding of XPC-hHR23B to UV-damaged DNA in the absence of non-damaged DNA showed only moderate preference for damaged DNA (Guzder *et al.*, 1998; Jansen *et al.*, 1998; Wakasugi and Sancar, 1999).

There is current discussion as to whether XPA or XPC functions first in the recognition of damage or helix distortion. Both RPA and XPA preferentially bind to cisplatin- or UV-damaged DNA (Jones and Wood, 1993; He *et al.*, 1995; Lee *et al.*, 1995; Li *et al.*, 1995; Burns *et al.*, 1996; Patrick and Turchi, 1998) and form a stable complex on damaged DNA (Wang *et al.*, 2000). Interaction between XPA and RPA is not only crucial for damage-recognition but also for recruiting other repair factors such as XPG, ERCC1-XPF, and TFIIH to

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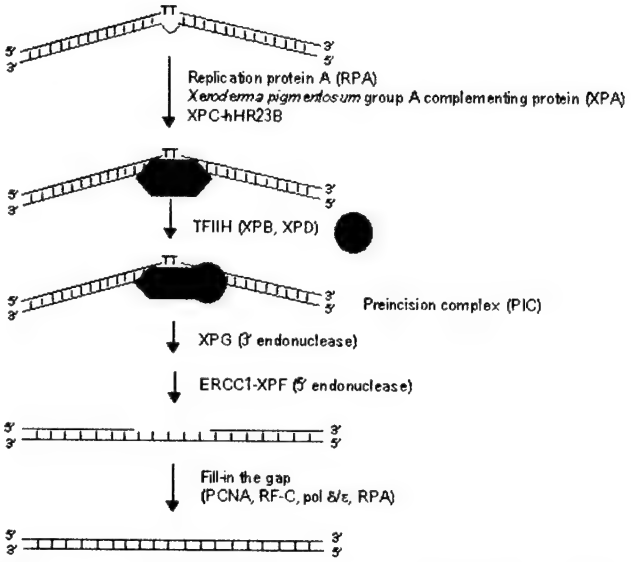

Fig. 1. A model for the nucleotide excision repair of UV-damaged DNA.

the damaged site (He *et al.*, 1995; Park *et al.*, 1995; Nocentini *et al.*, 1997; Bessho *et al.*, 1997; de Laat *et al.*, 1998). The XPA-DNA interaction is relatively weak and characterized by rapid dissociation, whereas RPA formed a 100-fold more stable complex with UV-damaged DNA (Wang *et al.*, 2000). A mutant RPA lacking XPA interaction domain leads to destabilization of XPA-damaged DNA complex, implicating a role for RPA in the early stage of DNA repair.

XPE (also known as a UV damage-specific DNA binding protein (UV-DDB) is another UV-damage recognition protein found in human and monkey cells (Payne and Chu, 1994; Nichols *et al.*, 2000). XPE binds to various damaged DNA induced by UV, cisplatin, depurination, and nitrogen mustard (Payne and Chu, 1994). Recent *in vivo* and *in vitro* studies showed that UV-DDB stimulates the excision of cyclobutant pyrimidine dimer, but not (6-4) photoproducts (Wakasuki *et al.*, 2001). However, XP-E mutant cells show the mildest NER defect among the XP groups and an *in vitro* NER reaction can be reconstituted without XPE (Aboussekhra *et al.*, 1995; Kazantsev *et al.*, 1998; Nichols *et al.*, 2000), suggesting that XPE may not be an essential factor for NER. In addition to the repair factors described above, high mobility group (HMG) domain protein and human Ku autoantigen (Ku70-Ku80 complex, a regulatory subunit of DNA-dependent protein kinase) bind to cisplatin-DNA adducts (Hughes *et al.*, 1992; Turchi and Henkels, 1996), although they are not essential for NER activity. The binding of HMG protein to cisplatin-damaged DNA inhibited an *in vitro* excision repair activity, suggesting that HMG domain protein may influence DNA repair *in vivo* (Huang *et al.*, 1994).

DNA structure requirements

NER machinery is responsible for recognizing and removing most of the chemically modified bulky adducts, whereas non-bulky base adducts such as those caused by hydrolysis, oxygen free radicals, and simple alkylating agents are recognized and repaired by the base excision repair (BER). In NER, presence of non-complimentary bases alone does not cause oligonucleotides excision. The loss of hydrogen bonding between two helical strands is an essential signal for the recruitment of NER enzymes. Oligonucleotides excision also requires chemical modification of DNA. For example, cisplatin covalently binds to the N7 positions of guanine and adenine bases and mainly forms 1, 2-intrastrand d(GpG) or d(ApG) adducts. The 1,3-intrastrand d(GpTpG) cisplatin is more structurally distorted than 1,2-intrastrand adducts and is therefore recognized and repaired more efficiently (Bellon *et al.*, 1991; Patrick and Turchi, 2001). Inclusion of non-complementary residue opposite the 1, 2-intrastrand d(GpG) adducts also increases the structural distortion of DNA and also increases repair efficiency significantly (Moggs *et al.*, 1997; Mu *et al.*, 1997). These findings suggest that local disruption of Watson-Crick base pairing as well as chemical modification of bases are indispensable components of the molecular signal that attracts human NER enzymes to the damaged DNA sites (Buschta-Hedayat *et al.*, 1999).

The cisplatin-DNA adduct containing 1, 2-intrastrand d(GpG) cross-link not only generates structural distortion of DNA, but also induces DNA unwinding and DNA bending (>40°) toward the major groove (Rice *et al.*, 1988). All these changes in DNA structure are likely important for damage recognition in mammals since XPA binds most efficiently to rigidly bent duplexes but not to single-stranded DNA (Missura *et al.*, 2001), while RPA preferentially recognizes single-stranded DNA site rather than bent DNA. The association of XPA with RPA may generate dual sensorship that detects both backbone and base pair distortion of DNA. Since the affinity of XPA for bent duplexes is not compatible with its function in recognition of nucleotide lesions, XPA in collaboration with RPA may be involved in monitoring the integrity of the Watson-Crick base-pairing to verify the damage-specific localization of repair complexes (Buschta-Hedayat *et al.*, 1999; Missura *et al.*, 2001).

Damage recognition proteins

Requirement of multiple damage recognition factors in NER indicates that XPC-hHR23B, XPA, RPA, and TFIIH may all have unique role in damage recognition and the early stage of repair. It is still in debate however how these damage recognition proteins function at the damaged DNA site. XPA is a multifunctional zinc-finger protein involved in the damage recognition step of NER (Robins *et al.*, 1991; Guzder *et al.*, 1993; Jones *et al.*, 1993). Its zinc-finger motif is essential for its DNA binding activity as well as its function in NER (Matsuda *et al.*, 1995). XPA preferentially binds to (6-4) photoproduct of UV-irradiated DNA, although it exhibits a low affinity binding to UV-damaged DNA (Wang *et al.*,

Table 1. Mammalian proteins involved in early stage of nucleotide excision repair

Repair factor	(yeast homolog)	Size (kDa)	Role in repair
XPA	(RAD14)	31-kDa	Damage recognition, Interaction with RPA
RPA	(RFA1, 2, 3)	70, 34,11-kDa	Damage recognition, DNA binding
			Interaction with XPA
XPC-hHR23B	(RAD4)	125-kDa (XPC)	Damage recognition, DNA binding
	(RAD23)	58-kDa (HR23B)	Stabilzation of XPC, control of DNA repair?
TFIIH	(RAD25)	89-kDa (XPB)	DNA helicase
	(RAD3)	80-kDa (XPD)	DNA helicase
	(TFB-1)	62-kDa	Core TFIIH subunit
	(SSL1)	44-kDa	Core TFIIH subunit
	(KIN28)	41-kDa (Cdk7)	cyclin-dependent protein kinas
	(CCL1)	38-kDa	cyclin H
		34-kDa (CAK)	Cdk activating kinase
XPF	(RAD1)	112-kDa (XPF)	5'-incision
	(RAD10)	33-kDa (ERCC1)	5'-incision
XPG	(RAD2)	135-kDa (ERCC5)	3'-incision

2000). XPA's binding to damaged DNA is stabilized through interaction with RPA on damaged DNA (Stigger *et al.*, 1998; Wang *et al.*, 2000). XPA also plays a role in recruiting TFIIH to the damaged site for subsequent incision/excision step (Park *et al.*, 1995; Nocentini *et al.*, 1997).

RPA is a heterotrimeric protein complex consisting of 70-, 34-, and 11-kDa subunits involved in DNA replication, repair, and recombination (Wold, 1997). RPA is a 4-cysteine type zinc-finger protein and a mutation at any zinc-finger cysteine abolishes its function in repair (Stigger *et al.*, 1998; Dong *et al.*, 1999), suggesting a unique role for the zinc-finger domain in early stage of repair. In contrast to XPA, RPA's zinc-finger domain is not essential for DNA binding activity, but is involved in regulation of RPA's DNA binding activity through redox change [Lin *et al.*, 1998; Park *et al.*, 1999). RPA preferentially binds to UV-damaged DNA and its interaction with UV-irradiated DNA was not affected by prior enzymatic photoreactivation of DNA, suggesting a preferred binding of RPA to the (6-4) photoproduct (Burns *et al.*, 1996). It is interesting to note that RPA, unlike XPA, may bind to duplex cisplatin-damaged DNA via generating single-stranded DNA at the lesion (Patrick *et al.*, 1999). The findings that RPA stimulates the XPA-DNA interaction through interaction with XPA on damaged DNA and also interacts with two endonucleases, XPG and ERCC1-XPF, suggest that the XPA-RPA complex, once formed on damaged DNA, recruits together XPG, ERCC1-XPF, and TFIIH to the damaged site for subsequent incision/excision step (He *et al.*, 1995; Matsunaga *et al.*, 1996). RPA may also be involved in a later stage of NER, gap-filling, that requires PCNA, RF-C, and DNA polymerase δ (or ε) (Aboussekhra *et al.*, 1995).

XPC-HR23B is a human homolog of yeast Rad4 and Rad23 proteins, respectively, and has been shown to exhibit strong affinity for damaged DNA (Sugasawa *et al.*, 1998; Batty *et al.*, 2000), as does the yeast counterpart, Rad4-Rad23 complex (Jansen *et al.*, 1998). The 106-kDa XPC protein forms a stable complex with 43-kDa HR23B (Masutani *et al.*, 1994; Shivji *et al.*, 1994). In yeast, Rad23 without Rad4 does not show any DNA binding under conditions where Rad4-Rad23 complex does (Guzder *et al.*, 1998), suggesting that Rad4 may be responsible for recognition of UV-damaged DNA. Rad23 (HR23B) is essential for XPC function in NER and may be necessary for the solubility of Rad4 (Guzder *et al.*, 1998). Recent studies also indicate that HR23B may be involved in regulation of repair activity. For example, Rad23 contains ubiquitin-associated (UBA) domain that may play a role in controlling NER through proteosome-mediated ubiquitin-dependent degradation of repair factors (Gilletts *et al.*, 2001; Chen *et al.*, 2001). HR23B also interacts with base excision repair factor, MPG, suggesting that HR23B may have a unique role in DNA repair accommodating various repair pathways (Miao *et al.*, 2000).

Evidence suggests that XPC-HR23B is involved in the recruitment of TFIIH to the damaged site through physical interaction with TFIIH (Yokoi *et al.*, 2000). XPC-HR23B may collaborate with XPA in recruiting TFIIH since XPA also interacts with TFIIH on damaged DNA (Park *et al.*, 1995; Nocentini *et al.*, 1997). TFIIH, once loaded on the damaged DNA, may play a role in distinguishing the damaged strand from the non-damaged one since the translocation along a DNA strand by TFIIH DNA helicases is hampered by encountering a lesion (Naegeli *et al.*, 1992; Naegeli *et al.*, 1993). Local unwinding of the damaged DNA region by TFIIH likely generates junction between single-stranded DNA and duplex DNA that is recognized by two structure-specific endonucleases, XPG and ERCC1-XPF, for dual incision on the damaged strand.

Interaction of damage recognition proteins with DNA lesions

Both RPA and XPA preferentially bind to DNA lesions induced by cisplatin or UV-irradiation (Jones *et al.*, 1993; Guzder *et al.*, 1993; Asahina *et al.*, 1994; He *et al.*, 1995; Burns *et al.*, 1996; Wagasuki *et al.*, 1999; Wang *et al.*, 2000). RPA binds to (6-4) photoproduct-containing DNA with nearly 2-fold higher affinity relative to *cis-syn* cyclobutane dimer and nearly 4-fold greater than non-damaged DNA (Wang *et al.*, 2000). Although XPA showed preferential binding to (6-4) photoproduct-containing DNA, its interaction with damaged DNA was significantly weaker ($K_D = 2.13 \times 10^{-8}$ M) than RPA (2.02×10^{-10} M). Real-time analysis showed that XPA quickly dissociated from damaged DNA, while RPA remained associated with damaged DNA (Wang *et al.*, 2000; Wang *et al.*, 2001). Low affinity binding of XPA to damaged DNA is stabilized by RPA. A mutant RPA lacking XPA interaction domain failed to stabilize XPA-damaged DNA interaction, indicating that a role for RPA in NER may be to stabilize the interaction between XPA and damaged DNA through protein-protein interaction. Interaction of XPA with RPA on damaged DNA is not only crucial for damage-recognition and but may also play a role in subsequent steps in recruiting other DNA repair proteins such as XPG, ERCC1-XPF, and TFIIH to the damaged site (He *et al.*, 1995; Park *et al.*, 1995; Lee *et al.*, 1995; Li *et al.*, 1995; Saijo *et al.*, 1996; Stigger *et al.*, 1998).

The fact that both XPA and RPA showed only moderate preference to damaged DNA over non-damaged DNA raises a question as to whether XPA and/or RPA are responsible for recognition of damaged DNA *in vivo*. XPC-hHR23B showed a remarkable preference to UV-damaged circular DNA particularly in the presence of non-damaged competitor DNA (Sugasawa *et al.*, 1998; Sugasawa *et al.*, 2001). Preferential binding of XPC-hHR23B to damaged circular DNA was likely due to its higher binding affinity to UV-damaged DNA than to a non-damaged counterpart, but is also possible that high affinity binding may be due to structural alteration caused by UV-damage on circular DNA, but not on duplex DNA fragment. It is interesting that XPC-hHR23B, like RPA, physically interacts with XPA and stabilizes the XPA-damaged DNA interaction (Wang *et al.*, 2001). In fact, XPC-hHR23B and RPA exhibited remarkably similar biochemical properties: their binding to damaged DNA, physical interaction with XPA, and stabilization of XPA on damaged DNA. Nonetheless, preincubation of damaged DNA with XPC-hHR23B but not with RPA significantly enhanced XPA-damaged DNA interaction, suggesting that damage recognition complex may be formed in an ordered process, such that XPC-hHR23B interacts with damaged DNA before addition of XPA and RPA to form a stable damage recognition complex (Sugasawa *et al.*, 2001). A recent immunohistochemistry study also strongly supports a role for XPC as a global initiator in repair (Volker *et al.*, 2001) while suggesting a role for XPA and RPA as repair mediator proteins. Biochemical basis for the interaction of XPC and damaged DNA needs to be addressed to clarify the discrepancy.

It is highly speculated that three damage recognition proteins (XPA, RPA, and XPC-hHR23B) form a complex at the damaged DNA site, although there is no direct evidence on that. A potential implication of forming damage recognition complex on damaged DNA is to position the preincision machinery for dual incision reaction at damaged DNA strand. In DNA replication, origin binding protein such as SV40 T-antigen form a multiprotein complex with RPA and DNA polymerase (pol) α-primase complex for the proper positioning of pol α-primase to initiate DNA synthesis at the specific site (Hurwitz *et al.*, 1990; Bullock *et al.*, 1991). Likewise, the formation of multiprotein complex at damaged site may be necessary for proper positioning of two endonucleases XPG and ERCC1-XPF for accurate incision at the 3' and 5' sites of the damaged strand (de Laat *et al.*, 1998).

Protein-induced structural distortion of damaged DNA

In DNA replication, binding of origin recognition factor(s) to the replication origin leads to a significant structural distortion at AT-rich region, which is necessary for loading of DNA helicase(s) (Borowiec *et al.*, 1990 and the references therein). During NER, distortion of damaged DNA may also be essential for loading of two DNA helicases (XPB and XPD of the TFIIH complex) to the damaged site. Structural distortion at damaged DNA is not only crucial for the formation of preincision complex (PIC), but also necessary for dual incisions. Since the formation of PIC at damaged site requires RPA, XPA, XPC-hHR23B, and TFIIH in the expense of ATP hydrolysis (Evans *et al.*, 1997a; Evans *et al.*, 1997b; Mu *et al.*, 1997a), it is conceivable that multiple repair factors contribute to a structural distortion of damaged DNA. RPA is a leading candidate for structural distortion of damaged DNA. RPA's zinc-finger motif is essential for its function in NER (Stigger *et al.*, 1998). The failure of a zinc-finger mutant in supporting NER was not due to its role in stabilization of XPA-damaged DNA complex (Park *et al.*, 1999; Wang *et al.*, 2000), suggesting that RPA may have a unique role in early stage of repair. Tryptic digestion pattern and far-UV CD spectra analysis indicated that RPA undergoes a significant conformational change upon binding to DNA in its zinc-finger motif-dependent manner (Wang *et al.*, 2001). RPA's zinc-finger motif may mediate the transition of RPA-XPA interaction into a stable RPA-XPA-damaged DNA complex through its conformational change (Park *et al.*, 1999; Wang *et al.*, 2001). Alternatively, both RPA and XPA, two zinc-finger proteins, undergo conformational changes upon binding to the damaged DNA (Wang *et al.*, 2001) to further distort the damaged DNA. It is also interesting to note that requirement for XPC-hHR23B in dual incisions can be bypassed by the use of a bubble-like DNA substrate containing a thymidine

dimer (Mu *et al.*, 1997b), suggesting that XPC-hHR23B may also play a role in inducing a structural distortion and/or local opening of the damaged DNA site.

Concluding Remarks

The NER machinery is primarily responsible for the repair of various intrastrand cross-linked DNA (Sancar, 1996; Wood, 1996), although some NER factors such as ERCC1-XPF may also participate in the repair of interstrand cross-linked damage (Kuraoka *et al.*, 2000; Mu *et al.*, 2000). Although the details of damage recognition process is still not clear, all three damage recognition factors, XPA, RPA, and XPC-hHR23B, play an essential role in damage recognition and recruiting other repair factors to the damaged site for dual incision. Protein-induced structural distortion at the damaged DNA is likely necessary for the loading of TFIIH complex (XPB and XPD helicases) to initiate unwinding of both 3'- and 5'-sides of DNA damage. Therefore, a new challenge in early stage of repair is to unravel the role of damage recognition factors in further helix destabilization at damaged DNA site.

Another future challenge is to understand how damage recognition factors effectively recognize DNA damage on chromatin as compared to that on naked DNA as the chromosomal DNA *in vivo* is assembled into a compact nucleosome structure. We do not know the answer, however, chromatin accessibility factors such as GADD45 may play a role in mediating the recognition of damaged DNA on chromatin (Carrier *et al.*, 1999). Also, various DNA damages induce chromatin modifications such as phosphorylation of histone H2AX and acetylation of histone H3 (Paull *et al.*, 2000; Brand *et al.*, 2001). These modifications would likely facilitate the access of damage recognition proteins and/or DNA repair machinery to lesions.

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Involvement of DNA-Dependent Protein Kinase in Regulation of Stress-Induced JNK Activation

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ABSTRACT

DNA-dependent protein kinase (DNA-PK) is composed of a 460-kDa catalytic subunit and the regulatory subunits Ku70 and Ku80. The complex is activated on DNA damage and plays an essential role in double-strand-break repair and V(D)J recombination. In addition, DNA-PK is involved in S-phase checkpoint arrest following irradiation, although its role in damage-induced checkpoint arrest is not clear. In an effort to understand the role of DNA-PK in damage signaling, human and mouse cells containing the DNA-PK catalytic subunit (DNA-PKcs proficient) were compared with those lacking DNA-PKcs for c-Jun N-terminal kinase (JNK) activity that mediates physiologic responses to DNA damage. The DNA-PKcs-proficient cells showed much tighter regulation of JNK activity after DNA damage, while the level of JNK protein in both cell lines remained unchanged. The JNK proteins physically associated with DNA-PKcs and Ku70/Ku80 heterodimer, and the interaction was significantly stimulated after DNA damage. Various JNK isoforms not only contained a DNA-PK phosphorylation consensus site (serine followed by glutamine) but also were phosphorylated by DNA-PK *in vitro*. Together, our results suggest that DNA damage induces physical interaction between DNA-PK and JNK, which may in turn negatively affect JNK activity through JNK phosphorylation by DNA-PK.

INTRODUCTION

CELLULAR REPONSES to various genotoxic stresses include cell-cycle arrest, DNA repair, and apoptosis, all of which occur through damage signaling pathways initiated from either DNA damage or the plasma membrane (Angel and Karin, 1991; Liu *et al.*, 1996). DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia-mutated gene (*ATM*), and the AT-related gene (*ATR*) are upstream components of damage signaling pathways and are linked to cell-cycle checkpoint arrest (Canman *et al.*, 1994; Lees-Miller *et al.*, 1995; Park *et al.*, 1999; Rathmell *et al.*, 1997). The c-Jun N-terminal kinase (JNK) pathway is most notably initiated from the plasma membrane; however, it is strongly induced on treatment with various DNA-damaging drugs, suggesting that the JNK signaling pathway mediates a physiologic response to DNA damage (Coso *et al.*, 1995; Devary *et al.*, 1991; Liu *et al.*, 1996; Minden *et al.*, 1995).

DNA-PK is a heterotrimeric enzyme composed of a 460-kDa catalytic subunit (DNA-PKcs) and the two regulatory subunits, Ku80 and Ku70. DNA-PKcs is a serine/threonine protein kinase and is recruited to DNA strand-break sites necessary to activate its kinase activity (Casciola-Rosen *et al.*, 1995; Finnie *et al.*, 1995; Gupta *et al.*, 1995; Hendrickson *et al.*, 1991; Lieber *et al.*, 1997; Peterson *et al.*, 1997; Ramsden and Gellert, 1998; Savitsky *et al.*, 1995). Studies with DNA-PK-defective mutant cells indicated that DNA-PK is essential in the repair of double-strand breaks (DSBs) and for V(D)J recombination (Blunt *et al.*, 1995; Hendrickson *et al.*, 1991; Jackson, 1997). Compared with their wildtype counterparts, DNA-PKcs-deficient cell lines were additionally sensitive to heat-induced apoptosis (Hendrickson *et al.*, 1991), suggesting a role for DNA-PK in anti-apoptotic responses (Gupta *et al.*, 1995; Hendrickson *et al.*, 1991; Muller *et al.*, 1998; Peterson *et al.*, 1995). *In vitro*, DNA-PK phosphorylates heat shock protein 90 (hsp90) and a number of DNA-binding proteins, including the Ku autoantigen, replication protein A (RPA), simian virus 40 large tumor anti-

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gen (SV40 Tag), the tumor suppressor protein p53, and several transcription factors (Sp-1, Oct-1, Fos, and serum response factor) (Lees-Miller *et al.*, 1990). The physiologic role of DNA-PK in phosphorylation of these proteins, however, is not clear.

The JNK/SAPK signaling pathway in response to genotoxic stress is thought to be largely a compensatory and protective response that positively regulates genes involved in cellular protective functions, such as glutathione-S-transferase (GST), quinone reductase, and MTIIA (Bergelson *et al.*, 1994; Litz-Jackson *et al.*, 1992; Pinkus *et al.*, 1996). On the other hand, persistent JNK activation may promote, rather than protect against, apoptosis in certain cells, although JNK/SAPK alone may not be sufficient to induce apoptosis (Sanchez-Perez *et al.*, 1998; Widmann *et al.*, 1997). Apoptosis signal-regulating kinase (ASK1) has been identified as a mitogen-activated protein kinase (MAPK) kinase kinase (MAPKKK) that activates the JNK and p38 MAP kinase pathways (Ichijo *et al.*, 1997). It is also required for TNF-α-induced apoptosis; however, whether JNK activation is directly involved in this apoptotic pathway remains controversial (Faris *et al.*, 1998; Guo *et al.*, 1998; Roulston *et al.*, 1998). In addition, the possible involvement of the JNK/SAPK signaling pathway, such as the MAPK cascades, in cell proliferation and control of DNA metabolism has been suggested (Auer *et al.*, 1998; Potapova *et al.*, 1997). At present, a role for the JNK signaling pathway in cellular proliferation, DNA replication, or apoptosis has yet to be defined.

The JNK protein was initially characterized as a MAPK family member that binds and phosphorylates c-Jun at serines 63 and 73 located within its transactivation domain (Angel *et al.*, 1988; Kyriakis *et al.*, 1994, Pulverer *et al.*, 1991, Smeal *et al.*, 1991). Phosphorylation of these serine sites resulted in increased transactivation potential of c-Jun (Derijard *et al.*, 1994; Smeal *et al.*, 1991). The substrates of JNK have now been extended to include other transcription factors, such as ATF-2 and ELK-1 (Gupta *et al.*, 1995, 1996; Livingstone *et al.*, 1995). Ten JNK isoforms have been identified from human brain, and two main forms, of 46 kDa and 55 kDa, correspond to alternatively spliced variants of JNK1, JNK2, and JNK3 (Davis, 1994; Gupta *et al.*, 1995). The activity of JNK is regulated by dual phosphorylation of specific threonine and tyrosine residues by MKK4 (otherwise known as SEK1) (Duyster *et al.*, 1995; Jackson and Jeggo, 1995; Johnson *et al.*, 1996). Activation of JNK has been shown to occur in response to various types of external stress such as heat shock, inflammatory cytokines (Foltz *et al.*, 1998; Moriguchi *et al.*, 1997; Raingeaud *et al.*, 1995) and DNA-damaging agents: ultraviolet light, ionizing radiation, cisplatin (Liu *et al.*, 1996; Moriguchi *et al.*, 1997; Schreiber *et al.*, 1995) and mitomycin C (MMC; Liu *et al.*, 1996). Unlike p53 induction, JNK activation is not a universal response to DNA damage. Different genotoxic agents vary greatly in their ability to activate JNK yet are effective in inducing p53 accumulation, growth arrest, and eventual cell death.

Here, we report our investigation into the involvement of DNA-PK in the regulation of the JNK signaling pathway after genotoxic stress. We found that JNK activity in DNA-PKcs-proficient cells, in contrast to the activity in DNA-PKcs-deficient cells, was tightly regulated. In addition, our *in vitro* studies revealed that JNK protein associated with DNA-PK and was efficiently phosphorylated by DNA-PK, suggesting that DNA-PK plays an important role in controlling JNK activity in response to DNA damage.

MATERIALS AND METHODS

Proteins, chemicals, and antibodies

Polyclonal antibodies to JNK1 and JNK1/2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) or Pharmingen (San Diego, CA). The GST fusion form of c-Jun protein (GST-cJun; a kind gift from Dr. S. Boswell, Indiana University School of Medicine, Indianapolis, IN) containing residues 1 through 79 of human c-Jun was overexpressed in *Escherichia coli* and purified using glutathione-agarose affinity column chromatography. Purified GST-JNK2 (77-kDa) protein and GST-JNK1 expression plasmid were obtained from Santa Cruz Biotechnology. DNA-PK holoenzyme was purified from HeLa cells according to the procedure described previously (Lees-Miller *et al.*, 1990). The [γ-^{32}P]-ATP (4500 Ci/mmol) was obtained from ICN. Epidermal growth factor (EGF), Adriamycin (doxorubicin), MMC, and cisplatin were purchased from Sigma Chemical Co. (St. Louis, MO), and methylmethane sulfate (MMS) was obtained from Aldrich (Milwaukee, WI).

Cell culture and treatment conditions

Malignant glioblastoma cells, M059K (DNA-PKcs proficient) and M059J (DNA-PKcs deficient) (obtained from Dr. M. J. Allalunis-Turner, Cross Cancer Institute, Edmonton, Alb., Canada), were grown in tissue culture dishes in DMEM/F12 supplemented with 10% FBS at 37° C in a CO_2 incubator. Mouse cell lines CB-17 (DNA-PKcs proficient) and SCID (DNA-PKcs deficient) (obtained from Drs. J.M. Brown and C. Kirchgessner, Department of Radiation Oncology, Stanford University, Stanford, CA) were maintained in DMEM supplemented with 10% FBS. To see the effect of the EGF (20 ng/ml) and genotoxic agents, cells were treated with MMS (0.1 or 1.0 mM), MMC (0.1 mM), or Adriamycin (1.0 μg/ml) and incubated until harvest.

Cell preparation and JNK immune complex assay

Cells were grown in culture medium containing 0.5% FBS for 16 h prior to treatment with EGF or genotoxic agents. Cells were washed in ice-cold PBS, with addition of 0.5 ml of JNK lysis buffer (25 mM HEPES, pH 7.5; 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5% Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium vanadate, 0.1 μM okadaic acid, 1 mM phenylmethylsulfonyl fluoride, aprotinin 20 μg/ml, leupeptin 50 μg/ml, and 10 μM pepstatin to the dishes (150 × 25 mm) before harvest. After 30 min on ice, insoluble material was removed by sedimentation for 30 min at 12,000 × g. The JNK activity was determined by an immunocomplex assay essentially as described (Duyster *et al.*, 1995; Litz-Jackson *et al.*, 1992). Briefly, cell extracts (200 μg) were mixed with 1.5 μl of anti-JNK1/JNK2 polyclonal antibody, and 15 μl of protein A-Sepharose beads was added for further incubation at 4°C for 3 h. The immune complexes were washed three times with JNK lysis buffer (0.5 ml each time) and once with JNK reaction buffer (20 mM HEPES, pH 7.5; 10 mM

$MgCl_2$, 7 mM $MnCl_2$, 1 mM EGTA, 1 mM sodium fluoride, 1 mM sodium vanadate, and 1 mM DTT). The immunoprecipitate was then resuspended in 30 μl of JNK reaction buffer containing 2 μg of GST-c-Jun (Litz-Jackson *et al.*, 1992), 50 μM ATP; and the reaction was initiated by the addition of 1.0 μl of [γ-^{32}P]-ATP (45,000 Ci/mmol). After incubation for 20 min at 30°C, the reaction was terminated by the addition of 8 μl of 5 × SDS sample buffer (Laemmli, 1970) and heating to 95°C for 5 min. Samples were analyzed by 12% SDS-PAGE.

Coimmunoprecipitation and Western blot analysis

Cell extracts (500 μg) were incubated with the respective antibodies and protein A-Sepharose for 3 h at 4°C. Precipitates were washed three times with JNK lysis buffer, suspended in SDS sample buffer, boiled for 3 min, and subjected to SDS-PAGE on 6% or 10% gels. Following gel electrophoresis, proteins were transferred to a nitrocellulose membrane and immunoblotted with primary antibody followed by a peroxidase-coupled secondary antibody (Amersham) and an enhanced chemiluminescence reaction mixture (Amersham) prior to visualization on Kodak X-O-mat film.

In vitro *phosphorylation of JNK*

Purified DNA-PK holoenzyme or cell extracts were mixed with 20 μl of buffer containing 20 mM HEPES, pH 7.5; 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM EGTA, 0.125 mM ATP, 1.0 μg of substrate peptide (EPPLSQEAFADLWKK) representing amino acids 11 through 24 of p53 (Lees-Miller *et al.*, 1990), and 1.0 μl of [γ-^{32}P]-ATP (45,000 Ci/mmol) for 20 min at 30°C. Where indicated, 1.0 μg of activated calf thymus DNA (Sigma) was included in the reaction mixtures. Phosphorylation of protein was analyzed by 10% SDS-PAGE followed by autoradiography.

RESULTS

Stress-induced JNK activation is tightly regulated in DNA-PKcs-proficient cells

Although the JNK signaling pathway is largely responsible for the activity of genes involved in cellular protective functions, such as GST, quinone reductase, and MTIIA (Bergelson *et al.*, 1994; Litz-Jackson *et al.*, 1992; Pinkus *et al.*, 1996), continuous JNK activation may induce apoptosis in certain cells (Sanchez-Perez *et al.*, 1998; Widmann *et al.*, 1997), suggesting that regulation of JNK is important for cell protection against apoptosis. In contrast, the physiologic role of DNA-PK may be to protect cells from DNA damage because DNA-PK is involved in immediate S-phase arrest and may be necessary for the efficient repair of DNA damage (Anderson and Carter, 1996; Park *et al.*, 1999). DNA-PK may be directly involved in S-phase arrest, targeting replication

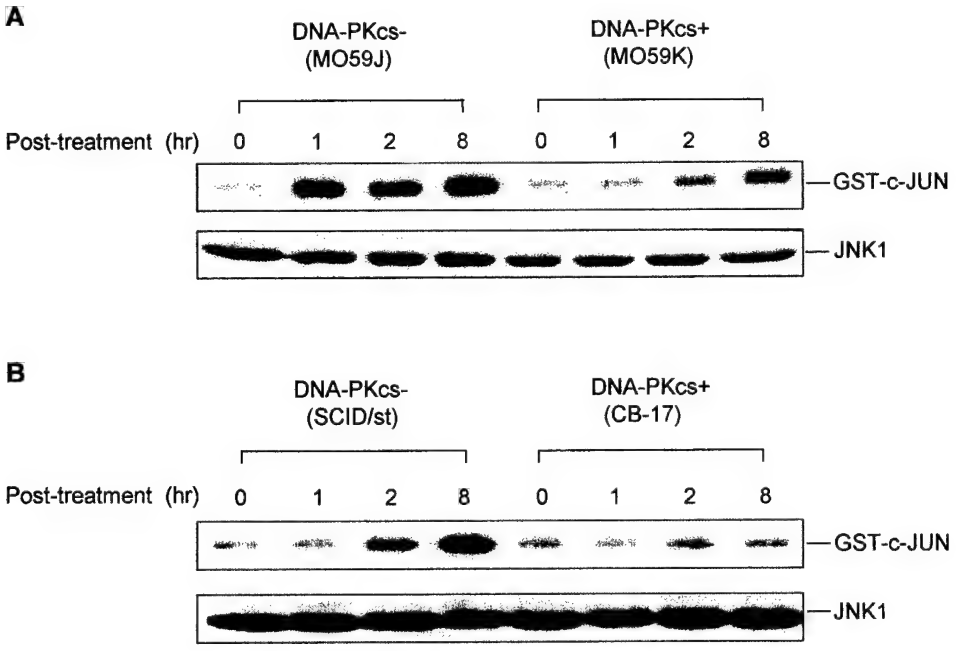

FIG. 1. Damage-induced JNK1 activation in DNA-PKcs-deficient and DNA-PKcs-proficient cells. Two human malignant glioblastoma cells (M059K [DNA-PKcs proficient] and M059J [DNA-PKcs deficient]) (**A**) or two mouse cell lines (CB-17 [DNA-PKcs proficient] and SCID-st [DNA-PKcs deficient]) (**B**) were treated with 0.1 mM MMS and harvested at times indicated. Cell lysates were examined for JNK activity using GST-c-JUN as a substrate (upper panel). The levels of JNK1 protein were also measured by immunoblot analysis using an anti-JNK1 antibody (lower panel). For JNK assay, 200 μg of cell lysates was mixed with an anti-JNK-1 polyclonal antibody and protein A-Sepharose beads and incubated with rocking for 3 h at 4°C. After the beads had been washed three times, the JNK immunocomplex was precipitated and used for an *in vitro* kinase assay in the presence of GST-cJun and [γ-^{32}P]-ATP. Phosphorylation of GST-JNK1 was analyzed by 10% SDS-PAGE and autoradiography.

factors after DNA damage (Park *et al.*, 1999), or it may participate in the damage signaling pathway, targeting protein kinases such as JNK.

In an effort to understand the role of DNA-PK in the damage signaling pathway, we examined the JNK activity of two human glioblastoma cells, M059K (DNA-PKcs proficient) and M059J (DNA-PKcs deficient), in response to genotoxic stress. Cells were treated with a DNA alkylating agent, MMS (0.1 mM), and examined for *in vitro* JNK activity using GST-cJun as a substrate (Fig. 1; upper panel) and JNK protein level (lower panel) following cell harvest at various time points (Fig. 1; panel A). Although JNK activity was induced by MMS treatment in both DNA-PKcs-proficient and DNA-PKcs-deficient, cells lacking DNA-PKcs showed remarkably greater activation than the DNA-PKcs-proficient cells (Fig. 1A). Amounts of JNK protein remained unchanged following genotoxic stress in both DNA-PKcs-deficient and DNA-PKcs-proficient cells, suggesting that the difference in JNK activity in the two cell lines is not at the level of gene expression.

Glioblastoma cells may carry multiple mutations; hence, the difference in JNK activity between DNA-PKcs-proficient (M059K) and DNA-PKcs-deficient (M059J) cells could also be attributable to mutations of factor(s) other than DNA-PK. Therefore, we also examined two mouse cell lines, SCID (DNA-PKcs deficient) and its parental line, CB-17 (DNA-PKcs proficient) (Blunt *et al.*, 1995; Hendrickson *et al.*, 1991), to see whether DNA-PKcs is responsible for downregulation of JNK activity. Similar to the result with human glioblastoma cells (Fig. 1), SCID cells showed significantly higher JNK activation after MMS treatment than did CB-17 cells (Fig. 2). Together, our results suggest that DNA-PKcs (or its holoenzyme) may be involved in controlling JNK activation in response to genotoxic stress.

Tight regulation of JNK activity in DNA-PKcs-proficient cells occurs only in the presence of DNA damage

DNK-PK gets activated only in the presence of DNA damage, whereas JNK activation can be induced either by DNA damage or by other agents such as EGF (Adler *et al.*, 1992; Foltz *et al.*, 1998; Roulston *et al.*, 1998). We therefore exam-

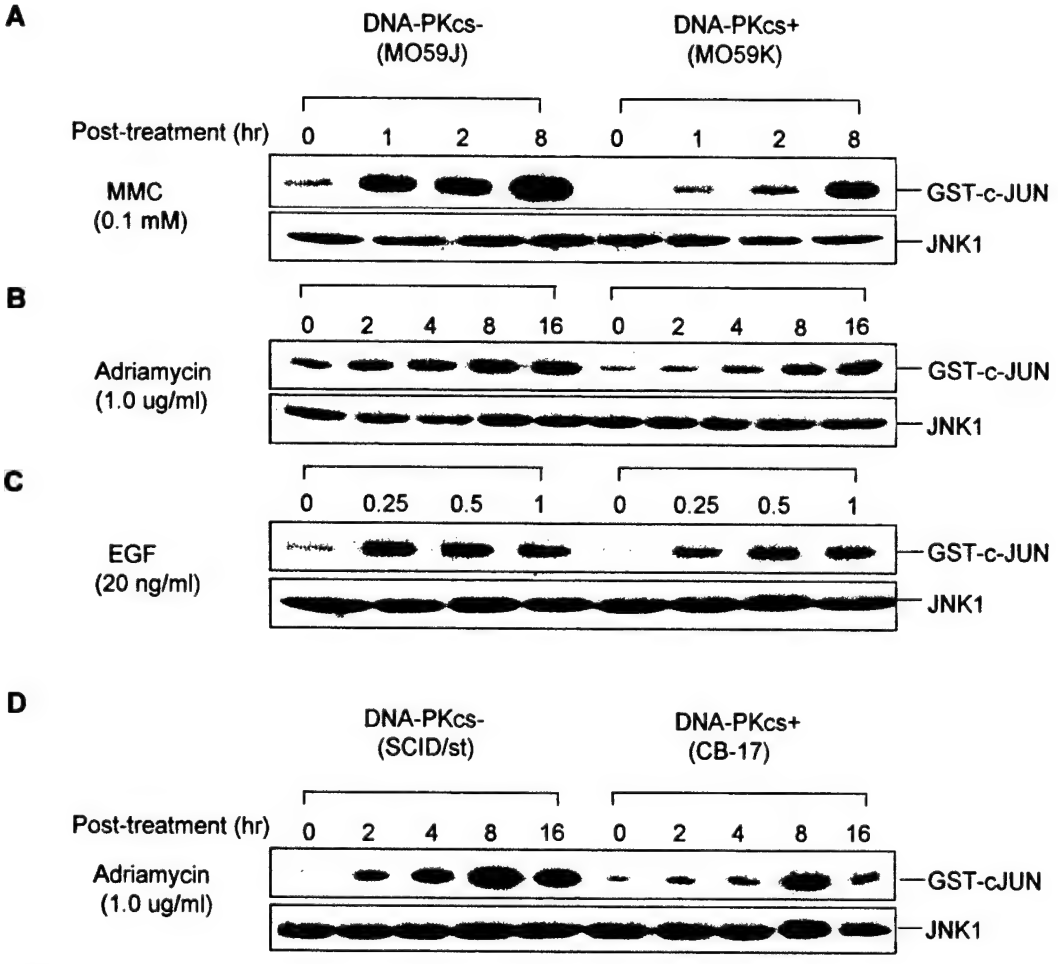

FIG. 2. Activation by JNK of DNA-PKcs-proficient and DNA-PKcs-deficient cells following the treatment with MMC, Adriamycin, or EGF. Human and mouse cells (DNA-PKcs proficient and DNA-PKcs deficient) were treated with 0.1 mM MMS (**A**), Adriamycin 1.0 μg/ml (**B, D**), or EGF 20 ng/ml (**C**) and harvested at times indicated. Cell extracts were examined for JNK activity using GST-c-Jun as a substrate (upper panel) and for JNK1 protein by Western blot analysis using an anti-JNK1 antibody (lower panel). The JNK immunocomplex assay was carried out as described in the legend to Figure 1.

ined whether tight regulation of JNK activity in DNA-PKcs-proficient cells occurs in a DNA damage-dependent manner. DNA-PKcs-proficient and DNA-PKcs-deficient cells were examined for JNK activation after treatment with DNA-damaging drugs or EGF. Similar to MMS treatment (see Fig. 1), DNA-PKcs-proficient cells (M059K) compared with the DNA-PKcs-deficient cells (M059J) showed significantly tighter regulation of JNK activity in response to treatment with the DNA-damaging drugs MMC or Adriamycin (Fig. 2A, B). In contrast, DNA-PKcs-proficient and DNA-PKcs-deficient cells showed similar JNK activation after EGF treatment (Fig. 2C), suggesting that damage-induced activation of DNA-PK may be necessary for regulation of JNK activity after treatment with DNA-damaging agents.

The JNK protein associates with DNA-PK in response to DNA damage

The results shown in Figures 1 and 2 strongly suggested that DNA damage-induced activation of DNA-PK is involved in regulation of JNK activity. Hence, we examined whether DNA-PK physically interacts with JNK in response to DNA damage. DNA-PKcs-proficient cells were treated with 0.1 mM MMS and harvested at various times. Cell extracts were then incubated with anti-JNK1/2 polyclonal antibody for immunoprecipitation of JNK1/2 protein and its associated proteins. Western blot analysis indicated that cells treated with MMS showed a marked increase in interaction of both DNA-PKcs and Ku70 with JNK1/2 (Fig. 3A, B). Levels of DNA-PKcs and Ku70 proteins remained unchanged, suggesting that the interaction between JNK1/2 and DNA-PK is altered by DNA damage.

Phosphorylation of JNK by DNA-PK in vitro

DNA-PK phosphorylates a number of proteins involved in transcription, replication, and DNA repair after DNA damage (Jackson and Jeggo, 1995; Lees-Miller *et al.*, 1990). Downregulation of JNK activity in DNA-PKcs-proficient cells may be secondary to the phosphorylation of JNK protein by DNA-PK

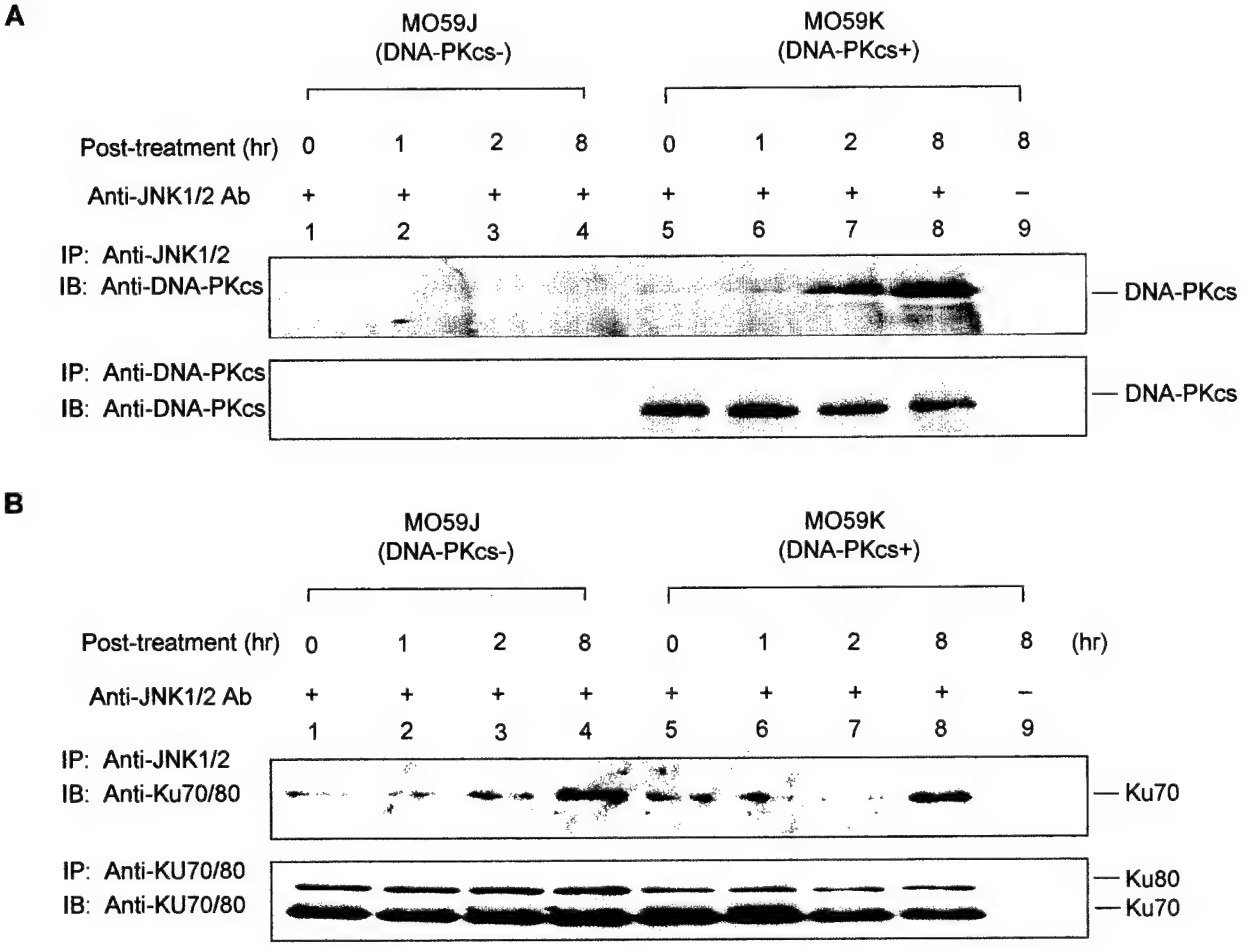

FIG. 3. *In vivo* association of JNK protein(s) with DNA-PKcs or Ku70/80. Two human malignant glioblastoma cells (M059K [DNA-PKcs proficient] and M059J [DNA-PKcs deficient]) were treated with 0.1 mM MMS and harvested at the indicated time points. (**A**) Cell lysates were subjected to immunoprecipitation with anti-JNK1/2 (upper panel) and anti-DNA-PKcs (lower panel). Immunoprecipitates were analyzed by immunoblotting with anti-DNA-PKcs. Lysates from DNA-PKcs-proficient glioblastoma cells were precipitated without antibody and used as a negative control (lane 9). (**B**) Cell lysates were used for immunoprecipitation of JNK1/2 with an anti-JNK1/2 antibody, and the precipitates were analyzed for coimmunoprecipitation of Ku70 by immunoblot using an anti-Ku70 antibody (upper panel). As a control experiment (lower panel), an anti-Ku70 antibody was also used for immunoprecipitation and Western blotting. No antibody was included in lane 9 during immunoprecipitation.

in response to DNA damage. At least 10 JNK isoforms have been identified (Gupta *et al.*, 1996), and all isoforms contain the conserved TPY consensus phosphorylation site for SEK1/MEKK4 that is required for JNK activation (Moriguchi *et al.*, 1997). All members of JNK kinase family also contain at least one consensus phosphorylation site (-SQ-) for DNA-PK (Anderson and Miller, 1992; Lees-Miller *et al.*, 1990; Fig. 4A), suggesting that JNK may actually be a substrate for DNA-PK. We therefore examined whether DNA-PK phosphorylates JNK protein *in vitro*. Purified DNA-PK was incubated with a GST fusion form of either JNK1 or JNK2 and examined for JNK phosphorylation. DNA-PK phosphorylated the GST fusion form of JNK1 (71 kDa) and JNK2 (77 kDa) in a DNA-dependent manner but did not phosphorylate GST alone (Fig. 4). This result suggests that the members of JNK family may be the actual substrates for DNA-PK *in vivo*.

We further examined whether DNA-PK is the major protein kinase responsible for JNK phosphorylation. A nonphosphorylated form of GST-JNK2 from *E. coli* was incubated with extracts of DNA-PKcs-proficient (M059K) or DNA-PKcs-deficient (M059J) cells as a source for protein kinase (Fig. 5). Phosphorylation of JNK2 (77-kDa GST-JNK2) was observed

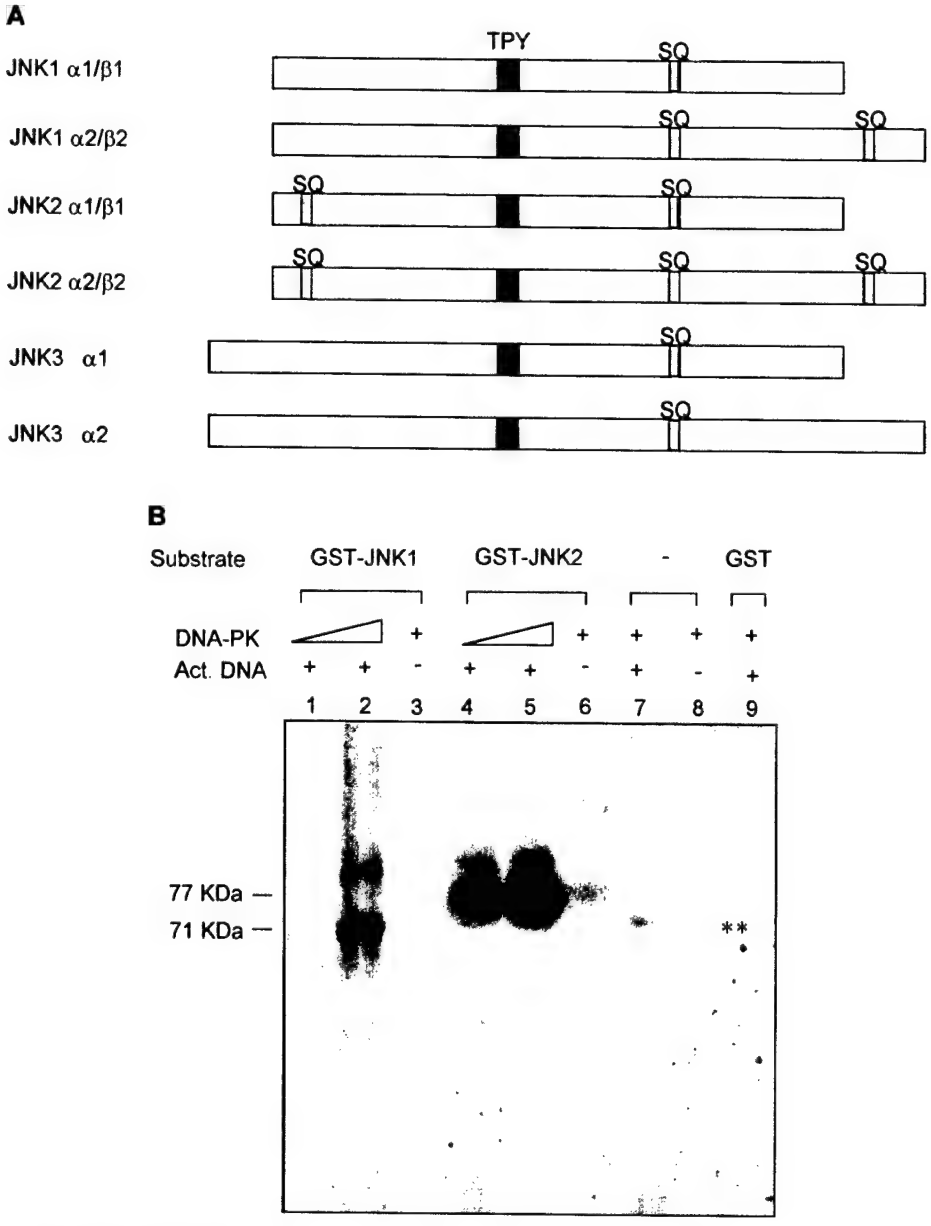

FIG. 4. *In vitro* phosphorylation of JNK proteins by DNA-PK. (**A**) DNA-PK phosphorylation consensus sites among members of the JNK kinase family. TPY represents the phosphorylation sites for MEK4/ SEK1 (or MKK7), and SQ represents the phosphorylation sites for DNA-PK. (**B**) *In vitro* phosphorylation of JNK 1 and JNK 2 by DNA-PK. Reaction mixtures contained either 0.05 μg (lanes 1 and 4) or 0.1 μg (lanes 2, 3, and 5–8) of purified DNA-PK holoenzyme. Where indicated, 1.0 μg of activated calf thymus DNA and 1.0 μg of substrate (GST-JNK1 [lanes 1–3], GST-JNK2 [lanes 4–6], or 1.0 μg of GST [lane 8]) were included. * and ** = Positions of phosphorylated Ku80 and Ku70 proteins, respectively. Detailed procedure for protein kinase assay is described in Materials and Methods.

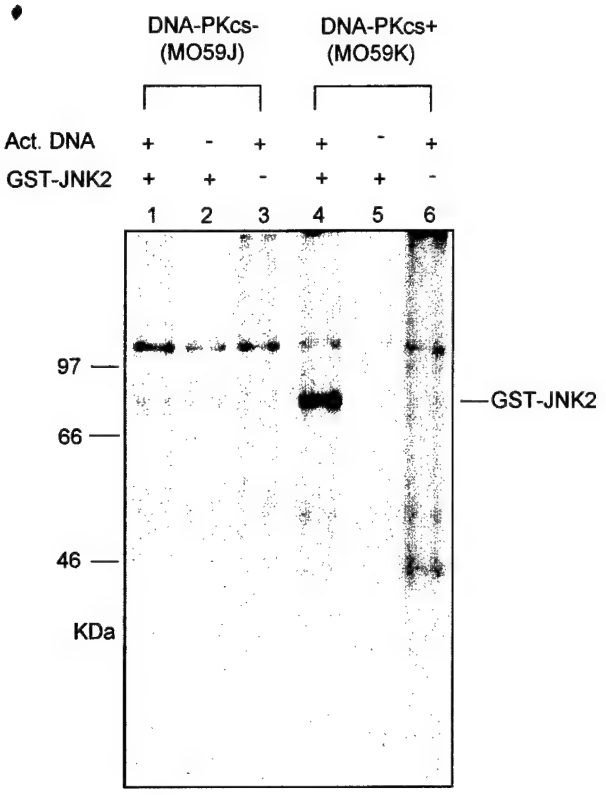

FIG. 5. *In vitro* phosphorylation of JNK2 in the presence of cell extracts from DNA-PKcs-proficient cells or DNA-PKcs-deficient cells. Cell extracts (5.0 μg) from DNA-PKcs-deficient (Mo59J; lanes 1–3) or DNA-PKcs-proficient (M059K; lanes 4–6) cells were used as the source of protein kinase. Reaction mixtures contained 20 mM HEPES, pH 7.5; 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$, 7 mM $MnCl_2$, 5 mM NaF, 1 mM Na_3VO_4, and 50 μM of [γ-^{32}P]-ATP (sp. act. 30,000 cpm/pmol). Where indicated, 1.0 μg of purified GST-JNK2 (77 kDa) or 1.0 μg of activated calf thymus DNA was included. After incubation for 30 min at 30°C, reaction mixtures were subjected to 10% SDS-PAGE; and, after drying, the gel was exposed to X-ray film. The position of GST-JNK2 is indicated at left.

only when it was incubated with extracts of DNA-PKcs-proficient cells, not with extracts of DNA-PKcs-deficient cells (Fig. 5), suggesting that DNA-PK is a major protein kinase responsible for JNK phosphorylation. Because DNA-PK is activated in response to DNA damage, we also examined JNK phosphorylation with extracts of nonirradiated and irradiated DNA-PKcs-proficient cells. Extracts of irradiated cells showed higher levels of JNK phosphorylation *in vitro* than did those of nonirradiated cells, suggesting that JNK phosphorylation by DNA-PK is induced in response to DNA damage.

DISCUSSION

The stress-induced signaling pathway involves a cascade of protein phosphorylation, including JNK/SAPK and c-jun (AP-1) phosphorylation, that enhances the transcription of factors involved in cellular protection, such as DNA polymerase, thiol synthesis, and DNA repair enzymes (Auer *et al.*, 1998; Bergelson *et al.*, 1994; Litz-Jackson *et al.*, 1992; Nishina *et al.*, 1997; Pinkus *et al.*, 1996; Potapova *et al.*, 1997). However, prolonged activation of JNK can lead to apoptosis (Auer *et al.*, 1998; Johnson *et al.*, 1995; Sanchez-Perez *et al.*, 1998; Widmann *et al.*, 1997), suggesting that the JNK/SAPK pathway may mediate either cell protection or programmed cell death after DNA damage. Hence, regulation of the JNK signaling pathway appears to be crucial for cell survival. The JNK/SAPK protein is activated by SEK1/MKK4 through the phosphorylation of the consensus site TPY that can also be dephosphorylated by MAP kinase phosphatases (MKPs). This implies a role for MKPs in blocking activation of the physiologic target of JNK/SAPK activation, the transcription factor c-Jun (Franklin *et al.*, 1998; Guo *et al.*, 1998b; Hirsh and Stork, 1997).

In this study, we found that DNA-PKcs-proficient cells in contrast to those lacking DNA-PKcs exhibited tight regulation of JNK/SAPK activation in response to genotoxic stress. This finding is in keeping with the previous observation that Ku70- or Ku80-deficient cells exhibit a significantly higher level of JNK than wildtype cells (Kim *et al.*, 1999). It is highly unlikely that the enhanced JNK activation in response to DNA damage in DNA-PKcs-deficient cells is reflective of the DNA repair deficiency of these cells, as there is no noticeable difference between DNA-PKcs-proficient and DNA-PKcs-deficient cells in nucleotide excision repair activity responsible for the removal of cisplatin or UV adducts (Park *et al.*, 1999). Regulation of JNK activity may occur *in vivo* through phosphorylation by DNA-PK, as both JNK1 and JNK2 were good substrates for DNA-PK *in vitro*. Our findings described here not only support a role for DNA-PK in the stress-induced signaling pathway but also imply a role for DNA-PK in protecting cells from apoptosis (Canman *et al.*, 1994; Lees-Miller *et al.*, 1995; Park *et al.*, 1999). The latter idea is further supported by previous observations that DNA-PK is the primary target for the CPP-32 (ICE-like) proteosome (Song *et al.*, 1996).

Activation of JNK occurs through phosphorylation by SEK1/MKK4 (or MKK7) in a dose (damage)-dependent manner (Derijard *et al.*, 1994; Devary *et al.*, 1991) and is critical for induction of cell-cycle arrest and DNA repair because high doses of genotoxic stress may lead to the apoptotic response. Stress-induced JNK activation is tightly regulated in DNA-PKcs-proficient cells compared with that observed in DNA-PKcs-deficient cells but only when cells were treated with low levels of irradiation or DNA-damaging agents (see Figs. 1 and 2). In contrast, significantly higher JNK activity was observed in both DNA-PKcs-proficient and DNA-PKcs-deficient cells treated with high doses of UV irradiation (50 J/m^2), MMS (1 mM), or cisplatin (1 mM) (data not shown). This result suggests that regulation of JNK by DNA-PK may be related to cell protection in response to physiologic doses of irradiation/DNA damaging agents.

Phosphorylation of JNK by SEK-1/MKK4 leads to the activation of JNK kinase, which then activates transcription factors (c-Jun, ATF-2, and ELK-1) through phosphorylation. The SEK-1/MKK4 phosphorylates TPY sites of JNK and activates its kinase. Following JNK activation, JNK kinase activity needs to be inactivated in a timely manner. Downregulation of JNK may occur through dephosphorylation of TPY sites by MKPs (Guo *et al.*, 1998a, b; Sanchez-Perez *et al.*, 1998); or, alternatively, direct phosphorylation of JNK by DNA-PK may also down-

regulate JNK activity. DNA-PK may selectively interact with JNKs/SAPK that is already phosphorylated at threonine and tyrosine residues (TPY sites) by MEKK4/7, as the interaction between JNK and DNA-PK is induced by DNA damage. However, it is also possible that DNA-PK phosphorylates JNK regardless of its phosphorylation status, which in turn alters JNK activity. A detailed *in vivo* analysis would be necessary to clarify the role of DNA-PK in regulation of JNK activity.

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Minireview

Molecules and Cells

DNA-dependent Protein Kinase Complex: a Multifunctional Protein in DNA Repair and Damage Checkpoint

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DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase that is activated upon DNA damage generated by ionizing radiation or UV-irradiation. It is a three-protein complex consisting of a 470-kDa catalytic subunit (DNA-PKcs) and the regulatory DNA binding subunits, Ku heterodimer (Ku70 and Ku80). Mouse and human cells deficient in DNA-PKcs are hypersensitive to ionizing radiation and defective in V(D)J recombination, suggesting a role for the kinase in double-strand break repair and recombination. The Ku heterodimer binds to double-strand DNA breaks produced by either DNA damage or recombination, protects DNA ends from degradation, orients DNA ends for re-ligation, and recruits its catalytic subunit and additional factors necessary for successful end-joining. DNA-PK is also involved in an early stage of damage-induced cell cycle arrest, however, it remains unclear how the enzyme senses DNA damage and transmits signals to downstream gene(s) and proteins.

Keywords: Damage Checkpoint; DNA Damage; DNA-PK; DSB Repair; Ku Complex; Nucleotide Excision Repair.

Introduction

DNA damage is constantly generated by radiation and chemotherapy drugs, which must be repaired to prevent genomic alterations that could otherwise contribute to cancer progression and/or generation of cancer. For this reason, cells invoke various mechanisms to repair DNA damage while operating damage checkpoint pathways that are responsible for sensing DNA damage and causing arrest of cell cycle progression until the damage is repaired. It is not clear, however, which genes are involved in this pathway or how DNA damage induces cell cycle arrest while permitting DNA repair. The DNA-dependent protein kinase (DNA-PK) is a member of the phosphatidylinositol-3 kinase (PI-3 kinase) family and shares amino acid sequence homology in its carboxy-terminal kinase domain with other family members, including ataxia telangiectasia mutated gene (ATM), ATM-related (ATR), and p110 PI-3 kinase (Hartley *et al.*, 1995; Poltoratsky *et al.*, 1995). All members of the PI 3-kinase family are activated by stress; PI-3 kinase is regulated by heat shock and DNA-damage, and ATM, ATR, and DNA-PK are activated by DNA damage (Gottlieb and Jackson, 1993; Lin *et al.*, 1997; Rotman and Shiloh, 1997; Yuan *et al.*, 1997). The DNA-PK is a unique damage-response element not only because it functions as a DNA damage sensor, but also it is a key player in various DNA repair pathways. In this review, we focus on recent advances on the DNA-PK in DNA repair and damage checkpoint pathways.

The DNA-PK complex and its regulation

Association of the DNA end-binding Ku70/Ku80 heterodimer with the 470 kDa serine/threonine kinase catalytic subunit forms the DNA-PK holoenzyme that is essential for double-strand break (DSB) repair by non-homologous recombination in mammalian cells. The DNA-PK catalytic subunit (DNA-PKcs) is a very abun-

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Abbreviations: ATM, ataxia telangiectasia mutated; DNA-PK, DNA-dependent protein kinase; DSB, double-strand break; JNK, c-Jun N-ternus protein kinase; NHEJ, non-homologous end joining; PCNA, proliferating cell nuclear antigen; PI-3 kinase, phosphatidylinositol-3 kinase; RPA, replication protein A.

dant nuclear protein in human cells and a very large polypeptide of 4127 amino acids giving a mass of 470 kDa (Gottlieb and Jackson, 1993). Its imaging structure indicates that DNA-PKcs has an open, pseudo 2-fold symmetric structure with a gap separating a crown-shaped top from a rounded base (Chiu *et al.*, 1998). With the exception of the 400 amino acids of the C-terminus, the amino acid sequence of DNA-PKcs shows little amino acid similarity to any protein in the databases. The C-terminus of DNA-PKcs are similar to proteins of the phosphatidylinositol (PI) 3-kinase superfamily (Hartley *et al.*, 1995; Poltoratsky *et al.*, 1995), both of which possess the DXXXXN and the DXG amino acid motifs that are found in protein kinase and are required for catalysis (Fig. 1). Despite the amino acid similarity with lipid kinases, DNA-PKcs acts as protein kinase, and no lipid kinase activity has been detected with purified DNA-PKcs or DNA-PK complex *in vitro* (Hartley *et al.*, 1995).

Ku complex, consisting of Ku70 and Ku80 subunits, serves as regulatory subunits for DNA-PK kinase activity essential for dsb rejoining (Kurimasa *et al.*, 1999). It is a very abundant nuclear protein, estimated at 4×10^5 molecules per cell and is present in vertebrates, insects, yeast, and worms (Mimori *et al.*, 1986). The Ku complex binds to DNA ends, nicks, gaps, and regions of transition between single and double-stranded structure (Blier *et al.*, 1993; Falzon *et al.*, 1993). *In vitro* photocross-linking of Ku protein bound to single DNA-strand ends showed that the preferred orientation is when Ku70 contacts the major groove and Ku80 contacts the minor groove (Yoo *et al.*, 1999). The crystal structure of the human Ku heterodimer revealed that Ku70 and Ku80 share a common topology and form a dyad-symmetrical molecule with a preformed ring that encircles duplex DNA (Walker *et al.*, 2001). The binding site can cradle two full turns of DNA while encircling only the central 3–4 base pairs. Surprisingly, Ku makes minimal contacts with DNA bases and with the sugar-phosphate backbone, however its ring structure nicely fits into major and minor groove contours in order to support its interaction with broken DNA ends and to recruit DNA-PKcs to the DNA (Walker *et al.*, 2001).

Regulation of DNA-PK activity While the activation of DNA-PK kinase requires DNA ends, its activity can be regulated by several mechanisms. Heterodimerization is not only essential for Ku-dependent DNA repair *in vivo*, but may be also a way to regulate DNA-PK activity. A central region within Ku80 is required for heterodimerization with Ku70. Evidences suggest that heterodimerization is required for the stabilization of Ku70 and Ku80. Each Ku subunit can translocate to the nucleus not only through its own nuclear localization signal but also through heterodimerization with each other, suggesting that the heterodimerization of these Ku subunits is important for their nuclear entry (Gell and Jackson, 1999; Koike *et al.*, 1999; 2001; Osipovich *et al.*, 1999). *In vitro* work showed that DNA-PK kinase activity involving DNA-PKcs controls Ku entry into DNA which may regulate DNA transactions including transcription in the vicinity of double-strand breaks (Frit *et al.*, 2000).

Phosphorylation of both DNA-PKcs and Ku subunits (Ku70 and Ku80) was observed *in vivo* and *in vitro*. Phosphorylation of either DNA-PKcs or Ku subunit resulted in inactivation of the serine/threonine protein kinase activity, suggesting that protein phosphorylation is a way to regulate DNA-PK protein kinase activity (Chan and Lees-Miller, 1996; Douglas *et al.*, 2001) and that the protein phosphatase responsible for reactivation *in vivo* is a PP2A-like enzyme (Douglas *et al.*, 2001). Post-translational regulation of DNA-PK kinase activity may also occur through modulation of Ku70/80 DNA-binding activity observed in human neoplastic tissues, suggesting a possible role for heterodimer activity in tumor development (Pucci *et al.*, 2001). Also, irradiation induces the expression of Ku70, but not Ku80, in both p53 and ATM-dependent manner, providing cells with means of assuring proper DNA repair and/or cellular response to DNA damage (Brown *et al.*, 2000).

Role in DSB repair

DSBs are deleterious and potentially lethal form of DNA damage because they physically disrupt the continuity of the genome. Accordingly, eukaryotic cells have evolved two distinct DSB repair pathways. One pathway is homologous recombination repair (HRR) that predominantly occurred in lower eukaryotes such as *S. cerevisiae* and is mediated by Rad51 and Rad52 family proteins. The other pathway is non-homologous end joining (NHEJ) that is a major DSB repair pathway in mammals, at least in G0 or G1-phase of the cell cycle (Lee *et al.*, 1997). The NHEJ machinery consists of the DNA-PK complex and a complex of XRCC4 and DNA ligase IV. Although both HRR and NHEJ require the Rad50/Mre11/NBS1 complex in yeast and presumably also in mammalian cells (Critchlow and Jackson, 1998), it is unclear how DSB repair proteins are coordinated at the damage site. The current model of DNA-PK complex activation by dsDNA break is based on the tenet that without DNA, DNA-PKcs is inactive and incapable of binding Ku complex (Suwa *et al.*, 1994). When a DSB is introduced, Ku binds to the DNA because of its high affinity for DNA ends. The binding of Ku elicits conformational changes that allow it to bind DNA-PKcs. Thus the presumed roles of Ku complex are to first bind to DNA and then recruit DNA-PKcs to the DNA. Ku may also serve as an alignment factor that not only increases NHEJ efficiency but also the accuracy. Furthermore, a secondary NHEJ activity is present in the absence of Ku, which is error-prone and possibly assisted by base

pairing interaction (Feldmann *et al.*, 2000). Upon the assembly of DNA-PK holoenzyme on DNA breaks, this DNA repair complex activates its serine/threonine protein kinase activity and phosphorylates target substrates that colocalize with it on the ends of broken DNA.

The assembly and activation of the DNA-PK complex at a DNA strand break is central to NHEJ, although the *in vivo* study suggests that Ku complex but not DNA-PKcs may be essential for DSB repair (Gu *et al.*, 2000). The Ku complex and DNA-PKcs bind to DNA ends and are capable of physically tethering two ends of DNA molecules (Cary *et al.*, 1997; Yaneva *et al.*, 1998). Low resolution structures of DNA-PKcs revealed that DNA-PKcs has a cage-like structure with channels and cavities within the interior of the structure (Chiu *et al.*, 1998; Leuther *et al.*, 1999). Nonetheless, it is still unclear how the Ku/DNA complex activates the kinase activity of DNA-PKcs during DSB repair. One hypothesis is that DNA-PKcs undergoes a conformational change upon association with the Ku/DNA complex and this conformational change accounts for the activation of kinase activity. The kinase activity associated with DNA-PK is needed for DSB repair *in vivo*, since expression of a kinase-dead form of DNA-PKcs fails to complement the radiosensitive phenotype of a mammalian cell line that lacks the DNA-PKcs protein (Kurimasa *et al.*, 1999). However, the physiological targets of DNA-PK *in vivo* are unknown. The DNA-PK complex can physically tether two ends of a DSB in close proximity *in vitro* suggesting the hypothesis that the DNA-PK complex acts as a scaffold to assemble the NHEJ pathway proteins at a DSB (Cary *et al.*, 1997). In support of this, Ku recruits XRCC4/LigaseIV to DNA termini (McElhinny *et al.*, 2000), and Ku binds to MRE11/Rad50/NBS complex (Goedecke *et al.*, 1999). In *S. cerevisiae*, NHEJ of plasmid DSBs requires Ku, Xrcc4, and DNA ligase IV, as well as Mre11, Rad50, Xrs2, and DNA damage checkpoint proteins. In *S. pombe*, however, DSB repair is dependent on Ku complex and DNA ligase IV, but does not require Rad32, Rad50 (the *S. pombe* homologues of Mre11 and Rad50, respectively) and checkpoint proteins for NHEJ, suggesting that NHEJ pathway in eukaryotes may occur in more than one mechanism (Manolis *et al.*, 2001).

Other repair pathways DNA-PKcs-deficient cells exhibited sensitivity not only to ionizing irradiation but also in response to chemotherapy drugs and were associated with lower nucleotide excision repair activity, suggesting that DNA-PK may be involved in the repair of various types of DNA damage (Britten *et al.*, 1999; Frit *et al.*, 1999). Also, studies with drug-resistant or drug-sensitive cancer cells suggested that higher levels of DNA-PK expression lead to a drug-resistant cells, whereas the low DNA-PK activity was associated with cells with drug-sensitive phenotype (Shen *et al.*, 1997) and was linked to cell death via the accumulation of damaged DNA. More than 20-fold overexpression of the catalytic subunit of DNA-PK (DNA-PKcs) was observed with a drug-resistant cancer cells (Muller and Salles, 1997; Shen *et al.*, 1997). The Ku complex also interacts with a key base excision repair enzyme, AP-endonuclease (Chung *et al.*, 1996), suggesting a possible involvement of DNA-PK complex in various DNA repair pathways. It is not clear however whether the Ku or DNA-PK complex has a direct role in these repair pathways.

Role in damage checkpoint

Upon DNA damage, DNA-PK phosphorylates a number of proteins, including p53, RNA-polymerase II, RPA, topoisomerases, hsp90, SV-40 large T antigen, and many transcription factors such as c-Jun, c-Fos, oct-1, sp-1, c-Myc, TFIID, and many more. Among them, p53 is one of the legitimate targets for damage-induced cell cycle arrest. The p53 deficiency prolongs the survival of DNA-PKcs-deficient cells harboring DNA damage by allowing the accumulation of aneuploid cells, suggesting that the DNA-PKcs mutation links to a p53-mediated DNA damage checkpoint (Guidos *et al.*, 1996). The *in vivo* role of DNA-PK in the transduction of the DNA damage signal to p53 remains unclear (Jimenez *et al.*, 1999; Woo *et al.*, 1998), however, DNA-PK along with ATM and ATR collectively detect DSBs and transmit this signal to p53 by phosphorylation. This phosphorylation dissociates p53 from its negative regulator, mdm2, which allows p53 undergo further modification and activate transcription of the genes responsible for cell cycle arrest such as p21waf1/cip1. Both control and DNA-PKcs-null murine embryonic fibroblast cells showed phosphorylation and accumulation of p53 in response to irradiation. However, the rise in p21cip1/waf1 and mdm2 was found to be delayed and attenuated in DNA-PK-deficient cells, which correlated in time with delayed onset of G1/S arrest by flow cytometric analysis, suggesting that loss of DNA-PK activity appears to attenuate the kinetics of p53 to activate downstream genes, implying that DNA-PK plays a role in post-translational modification of p53, without affecting the increase in levels of p53 in response to DNA damage (Kachnic *et al.*, 1999).

Another potentially important role for DNA-PK in damage signaling stems from a functional interaction with c-Abl in response to DNA damage (Kharbanda *et al.*, 1997; Kumaravel *et al.*, 1998). Ionizing radiation stimulates the binding of c-Abl to DNA-PK and induces the association of c-Abl with Ku antigen (Jin *et al.*, 1997a; Kharbanda *et al.*, 1997). DNA-PK phosphorylates c-Abl *in vitro* and activates its tyrosine kinase activity. Moreover, via a potential feedback mechanism, c-Abl phosphorylates DNA-PK, which can then no longer form a complex with DNA

(Kharbanda *et al.*, 1997). Furthermore Ku associates with c-Abl and p21cip1/waf1 after irradiation (Kumaravel *et al.*, 1998), and interestingly, the p21cip1/waf1 only transiently associates with Ku complex at the low dose of irradiation, suggesting a possible role for DNA-PK in the damage-checkpoint pathway to control downstream DNA metabolism. The DNA-PKcs and Ku70/Ku80 heterodimer also can associate with another stress-response protein, the c-Jun N-terminus protein kinase (JNK), and the interaction was significantly stimulated following DNA damage. DNA-PKcs-proficient cells compared to those lacking DNA-PKcs exhibited a tight regulation of JNK activation in response to genotoxic stress. Based on the observation that the DNA-PK phosphorylates JNK *in vitro*, it is possible that DNA-PK is involved in regulation of JNK signaling pathway in response to stress/DNA damage (Park *et al.*, 2001).

G1/S-phase arrest Mounting evidences point to the role of DNA-PK in damage-induced S-phase arrest. Both *in vivo* and *in vitro* studies suggested that DNA-PK kinase activity is necessary for damage-induced replication arrest and its reversal (Park *et al.*, 1999; Wang *et al.*, 1999; 2001). Damage-induced S-phase arrest was reversed *in vitro* by the addition of a DNA-PK inhibitor or by immunodepletion of DNA-PKcs, suggesting that DNA-PKcs may be directly involved in damage-induced S-phase arrest through modulation of replication protein(s) (Park *et al.*, 1999; Wang *et al.*, 1999). Replication protein A (RPA) is a trimeric, multifunctional protein complex involved in DNA replication, DNA repair, and recombination and is a likely target for DNA-PK in damage-induced S-phase arrest because phosphorylation of the RPA2 subunit is observed after exposure of cells to ionizing radiation. DNA-PKcs interacted directly with RPA, and causes RPA2 phosphorylation in response to DNA damage. Phosphorylated RPA has a higher affinity for nuclear structures than unphosphorylated RPA suggesting the modified protein may be involved in the regulation of DNA replication after DNA damage or in DNA repair. RPA phosphorylation is delayed in cells deficient in ATM or DNA-PK, suggesting that DNA-PK and ATM may cooperate to phosphorylate RPA after DNA damage to redirect the functions of the protein from DNA replication to DNA repair (Wang *et al.*, 2001). DNA-PK-mediated RPA phosphorylation requires ongoing DNA replication but is prevented by the cell-cycle checkpoint abrogator, suggesting that the replication fork-associated RPA, when encounters DNA double-strand end-associated DNA-PK, leads to RPA phosphorylation which may signal the presence of DNA damage to an S-phase checkpoint mechanism (Shao *et al.*, 1999). Phosphorylation of RPA in yeast is also dependent on the central checkpoint regulator Mec1p. Because RPA's ssDNA binding activity of the RPA heterotrimer and is required for DNA replication, repair and recombination, it is possible that phosphorylation of this subunit is directly involved in modulating RPA activity during the checkpoint response (Brush and Kelly, 2000).

DNA damage induces the change in other repair/replication proteins that also affects DNA repair and/or G1/S-phase cell cycle arrest. The proliferating cell nuclear antigen (PCNA) forms specific foci in response to DNA damage and an increased interaction of PCNA with the Ku heterodimer after DNA damage, suggesting a role for PCNA in the NHEJ repair pathway of DNA strand breaks (Balajee and Geard, 2001). Similarly, Ku80-deficient cells showed a significant decrease in the amount of PCNA associated with chromatin following ionizing radiation, suggesting that the Ku-complex by binding at the ends of damaged DNA may also protect the key replication factors from dissociating from chromatin (Park *et al.*, submitted). The Ku (or DNA-PK complex) also associates with ors8 and ors12 and this association was significantly enhanced in cells synchronized at the G1/S border. In addition, *in vitro* DNA replication activity with the use of extracts from Ku80-deficient cells was significantly lower compared to that with wild-type cell extracts, suggesting a novel function for Ku that may act at the initiation step of DNA replication and dissociates after origin firing (Novac *et al.*, 2001).

G2 arrest In addition to its role in damage-induced G1/S-phase arrest, DNA-PK may also have a distinct role in G2 checkpoint traversal in response to DNA damage since its activity is required for exit from a DNA damage-induced G2 checkpoint arrest (Lee *et al.*, 1997). This finding is supported by the observation that the activity of the DNA-PK complex is regulated in a cell cycle-dependent manner peaking its activity at the G1/ S boundary and at G2, which is suggestive of the involvement of DNA-PK at multiple checkpoints (Lee *et al.*, 1997). Ku80-deficient cells compared to wild-type cells were highly cytotoxic to various stress agents, which led to their long-term accumulation in the G2 phase. This differential response was not due to differences in DNA repair, since DNA damage was repaired with comparable efficiency in both wild-type and Ku80-deficient cells, but was associated with differences in the expression of important cell cycle regulatory genes, supporting the notion that Ku80-mediated cytoprotection and G2-progression are not only dependent on DNA repair but may also reflect its influence on other cellular processes (Arrington *et al.*, 2000).

Other DNA metabolism

Besides their role in DNA repair, Ku complex also binds to chromosome ends (telomeres) protecting them from

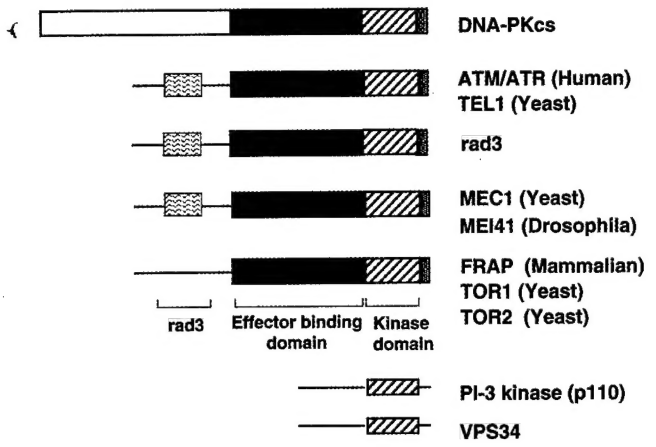

Fig. 1. DNA-PKcs and the related PI-3 kinase family among eukaryotes.

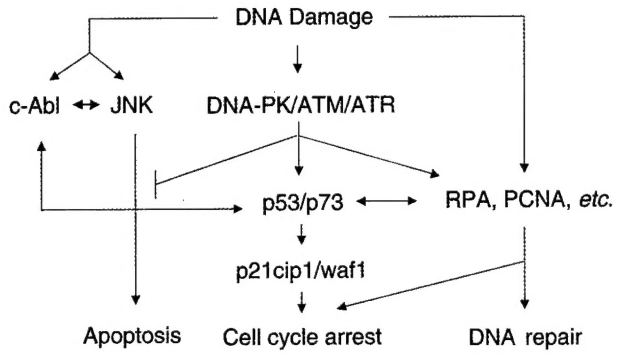

Fig. 2. Involvement of DNA-PK in damage-induced cell cycle arrest and DNA repair.

telomeric shorting and end-to-end fusions. Inactivation of Ku70 or Ku80 in mouse yields telomeric shortening in various primary cell types at different developmental stages and showed failure to proliferate in culture and show signs of premature senescence, suggesting that chromosomal instability of Ku-deficient cells results from a combination of compromised telomere stability as well as defective NHEJ (d'Adda *et al.*, 2001; Featherstone and Jackson, 1999). Localization of Ku to the telomere in yeast does not depend on the DNA-dependent protein kinase catalytic component, indicating that Ku complex not DNA-PK kinase activity is involved in telomere maintenance (Hsu *et al.*, 1999). In mammals, although not a significant change in telomere length or in deregulation of the G-strand overhang at the telomeres, DNA-PKcs cells display an increased frequency of spontaneous telomeric fusions and anaphase bridges, suggesting that DNA-PKcs or DNA-PK complex may have a role in telomeric end-capping (Bailey *et al.*, 1999; Goytisolo *et al.*, 2001; Song *et al.*, 2000). DNA-PK associates with the RNA polymerase I and II transcription complexes and likely negatively regulates them (Anderson, 1993; Bryntesson *et al.*, 2001; Chibazakura *et al.*, 1997; Kuhn *et al.*, 1995; Labhart, 1995; Peterson *et al.*, 1995). Ku recruits Werner syndrome protein (WRN) to DNA. Moreover, Ku complex stimulates and alters WRN exonuclease activity, suggesting that Ku-mediated activation of WRN exonuclease activity may play an important role in a cellular pathway that requires processing of DNA ends (Li and Comai, 2001). Ku also interacts with clusterin, a protein related to apoptosis signaling. Overexpression of nuclear clusterin in nonirradiated cells dramatically reduced cell growth with increased G1 checkpoint arrest and increased cell death, implicating a role for DNA-PK in controlling apoptosis (Jackson, 1996; Jin *et al.*, 1997b; McConnell and Dynan, 1996; Wang *et al.*, 2000; Yang *et al.*, 2000).

Concluding Remarks

DNA-PK is a unique protein kinase that is not only essential for DSB repair but also involved in damage checkpoint pathway in eukaryotes. For that reason, DNA-PK has long been a suspected factor involved in sensing and transmitting DNA damage signals to the downstream targets such as p53 and RPA, which eventually contribute to damage-induced cell cycle arrest and DNA repair (Anderson, 1993; Jackson, 1996). On the other hand, the observation that DNA damage induces the interaction of Ku complex with PCNA (Balajee and Geard, 2001) may indicate a potential role for DNA-PK in mediating DNA repair and replication in response to DNA damage (Fig. 2). The Ku complex seems to play a role in keeping PCNA and other replication proteins associated with the chromatin following DNA damage, which may be essential for subsequent DNA replication following the repair of damaged DNA and cell survival (Fig. 2; Park *et al.*, submitted).

JNK activation and its signaling pathway are linked to the induction of cell cycle arrest and DNA repair. Prolonged activation of JNK, however, can lead to apoptosis (Fig. 2; Auer *et al.*, 1998; Johnson *et al.*, 1996; Sanchez-perez *et al.*, 1998; Widmann *et al.*, 1997), suggesting that the JNK pathway may mediate either cell protection or programmed cell death following DNA damage. These lines of evidences implicate a role for DNA-PK in protecting cells from apoptosis (Canman *et al.*, 1994; Lees-Miller *et al.*, 1995; Park *et al.*, 1999). In this regard, it is obvious why DNA-PK is the primary target for the CPP-32 proteosome (Song *et al.*, 1996).

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